Effects of Dibutylphthalate on the Biosynthesis of Intermediates of the Androgen and Glucocorticoid Pathway in a Cultured Rat Leydig Cell Line (R2C)

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Abstract

Phthalate esters (phthalates) such as dibutylphthalate (DBP) are commonly used as plasticisers and pesticides in a variety of products such as children's plastic toys, food packaging, cosmetics, medical equipment (including surgical equipment), and acaricides. Because of their widespread use phthalates are ubiquitous environmental contaminants that humans are commonly exposed to. Phthalates are known endocrine-disrupting chemicals (EDCs) that are well known to cause male reproductive defects such as cryptorchidism (failed descent of the testes) and hypospadias (malformations in the urethra) in a range of different species if they are exposed *in utero*. They do this by reducing testosterone production in Leydig cells, which are the primary site of testosterone biosynthesis in the male. Because phthalates are dose-additive they are considered to share the same mechanism of toxicity. However, the details of phthalates mechanism of toxicity are not fully understood. The aim of this research was to investigate the effects of DBP on the steroidogenesis pathway using the cultured rat Leydig cell cancer line R2C as a Leydig cell model. R2C cells were exposed to a range of DBP concentrations (10 μg/mL, 5 μg/mL, 1 μg/mL, and 0.1 μg/mL) and their steroid hormone production was analysed using reverse phase HPLC. R2C cells did not synthesise testosterone at detectable levels. However, DBP exposure stimulated cortisol biosynthesis at all concentrations but caused no change in progesterone biosynthesis. This cortisol stimulation in Leydig cells has not been observed before. Because cortisol and testosterone compete for precursors an increase in cortisol synthesis could starve testosterone synthesis of precursors. On top of this it has been shown that glucocorticoids including cortisol have an adverse effect on Leydig cell development reducing steroid production and even causing apoptosis. This could explain how DBP and other phthalates can cause male developmental defects such as cryptorchidism and hypospadias.

List of Abbreviations

	Abbreviations
Fetal Leydig cells	FLCs
Adult Leydig cells	ALCs
Stem Leydig cells	SLCs
Progenitor Leydig cells	PLCs
Immature leydig cells	ILCs
3β-Hydroxysteroid dehydrogenase	3β-HSD
17β-Hydroxysteroid dehydrogenase	17β-HSD
Cytochrome P450	CYP
Desert Hedgehog protein	*Dhh*
Platelet-derived growth factors	PDGF
Steroidogenic factor 1	*Sf1*
5α-Dihydrotestosterone	DHT
Insulin-like factor 3	INSL3
Luteinizing hormone	LH
Steroidogenic acute regulatory protein	StAR
Follicle-stimulating hormone	FSH
Adrenocorticotropic hormone	ACTH
Cyclic adenosine monophosphate	cAMP
N^6,2-O-Dibutyryladenosine3',5'-cyclic monophosphate	DB-cAMP
Androgen receptor	AR
Estrogen receptor	ER
Endocrine disrupting chemical	EDC
Bisphenol A	BPA
Dichlorodiphenyldichloroethylene	DDE
dichlorodiphenyltrichloroethane	DDT
Phthalate diesters	Phthalates

Diethylhexylphthalate	DEHP
Dibutylphthalate	DBP
Monobutylphthalate	MBP
Monoethylhexylphthalate	MEHP
High performance liquid chromatography	HPLC
Fetal bovine serum	FBS
Phosphate buffered saline	PBS
Eagle's Minimum Essential Medium	MEM
PBS/EDTA solution	PE
Analytical grade diethylether	ether
Analytical grade methanol	MeOH
Limit of determination	LoD

Glossary

Cryptorchidism: The failure of one or both of the testes to descend into the scrotum during early development. Cryptorchidism is the most common birth defect regarding male genitalia.

Hypospadias: A birth defect resulting in the malformation of the urethra resulting in abnormal placement of the urethral orifice. Instead of opening at the tip of the penis it is anywhere along the line anywhere between the tip and the intersect between the penis and scrotum.

Androgens: The male sex hormones, responsible for the expression of male traits; comprises of 5 compounds, testosterone and 5α-dihydrotestosterone (DHT) are the primarily active hormones.

Estrogens: The female sex hormones, responsible for the expression of female traits; comprises of 3 compounds (estrone, estriol, and 17β-estradiol), 17β-estradiol is the primarily active hormone.

Corticosteroids: A class of chemicals that include steroid hormones produced in the adrenal cortex of vertebrates and analogues of these hormones synthesised in laboratories. Corticosteroids include the following steroid hormone groups: **Glucocorticoids** control carbohydrate, fat and protein metabolism as well as act as anti-inflammatories. Cortisol is the primary active glucocorticoid and is essential for a range of life functions. **Mineralocorticoids** control electrolyte and water levels. The primary mineralocorticoid is aldosterone which does this primarily by promoting Na^+ retention in the kidney.

Steroidogenesis: The entirety of the steroid biosynthetic metabolic pathway.

Small molecules such as steroid hormones and phthalates can exist in three forms when present in bodily fluids. The first is **free** in solution. This means the compound is in its original form or dissolved in the fluid and is bioavailable. The second is a **protein bound** form. In this state the compound is non-covalently bound to a carrier protein. The compound is sequestered in this state but will be readily released in response to equilibrium shifts in the fluid. The last is a **conjugated** form. In this state the compound will have a highly water soluble component such as a glucuronidate or sulphate covalently bound to it. Conjugated compounds are not bioavailable and are excreted.

Endocrine system: Consists of all the hormone secreting glands in an organism as well as the secreted hormones and their activity.

Hyperthyroidism: A condition where the thyroid gland produces excess amounts of thyroid hormones.

Agonist: A compound that has the ability to bind to a cell's hormone receptor and induce a biological effect.

Antagonist: A compound that can bind to a cell's hormone receptor but will not induce a biological response and will block the binding site preventing the endogenous hormone from binding, reducing the biological effect.

Epigenetic effects: Changes in gene expression or cellular phenotype that are caused by mechanisms that are DNA independent (e.g. DNA methylation, and histone modification).

Constitutive expression: A protein whose activity is constant and active and therefore is not regulated.

Chapter 1 - Introduction

1.1. The Biology and Metabolism of Leydig Cells

1.1.1. Where are Androgens Made? A Brief History of Leydig cells

Leydig cells were originally described by Franz von Leydig (1821-1908) in 1850 (Leydig 1850). His seminal article was primarily a comparative study of male reproductive histology in different species. When Leydig observed the testis he noticed clusters of cells found consistently on blood vessels between the seminiferous tubules (the site of spermatogenesis) these cells were observed in multiple species and would come to be known as Leydig cells (Fig. 1.1).

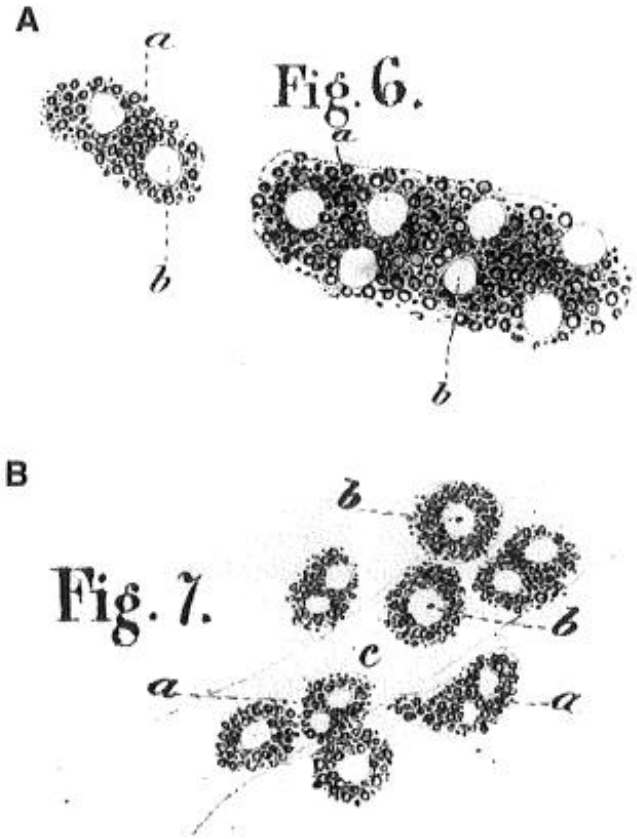

Figure 1.1: Illustrations of Leydig cells by Franz Leydig in his original 1850 article (Leydig 1850) *Figures 6 and 7 are the only ones of Leydig cells in his article. He described these as diagrams of "masses found between the seminiferous tubules and also found in the mediastinum of the cat testis (Figure 6) and the bat testis (Figure 7).* ***(a)*** *Small fat granules embedded in a soft ground substance.* ***(b)*** *The clear vesicular nuclei that are enclosed in the masses.* ***(c)*** *A blood vessel on which the masses are usually situated."*

In the late 19th century, better specimen preparation methods were developed allowing further elucidation of the Leydig cell superstructure. For example, Friedrich Reinke (1862-1919) was able to observe crystalloids (crystal like structures) in human Leydig cells (Reinke 1896).

During this period there were many suggestions as to what the function of the Leydig cell might be, but none of them considered they might have an endocrine function. The most popular of these, believed by Leydig himself, was that Leydig cells functioned as connective tissue cells. This was reinforced by a paper by David Hansemann (1858-1920) in 1895 (Hansemann 1895) which discussed the reproductive cycle of marmots (*Marmota marmota*). He showed that the annual atrophy of the Leydig cells gave rise to fibroblast-like cells in the interstitial tissue. The then most popular hypothesis for Leydig cell function was that Leydig cells took up nutrients from circulation, processed them, and then passed them on to the seminiferous tubules (Fig. 1.2) to aid in spermatogenesis.

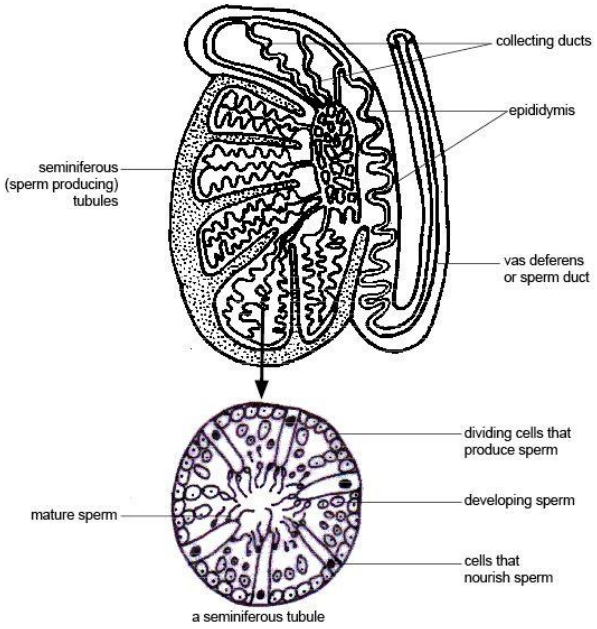

Figure 1.2: Diagram of a testis and seminiferous tubule. Sperm are produced in the many seminiferous tubules and transported to the vas deferens. Between the seminiferous tubules interstitial tissue is present and Leydig cells are a part of this interstitial tissue. (By Ruth Lawson at http://commons.wikimedia.org/wiki/File:Anatomy_and_physiology_of_animals_The_testis_%26_a_magnified_seminferous_tubule.jpg)

The first to strongly propose that Leydig cells had an endocrine function were Pol André Bouin (1870-1962) and Paul Ancel (1873-1961). Between 1897 and 1905 they conducted studies on Leydig cells and their endocrine role in regulating male secondary sexual characteristics. They published four major articles, one in 1903 (Bouin & Ancel 1903), two in 1904 (Bouin & Ancel 1904; Ancel & Bouin 1904), and one in 1905 (Bouin & Ancel 1905). Their first article (Bouin & Ancel 1903) describes Leydig cells in the pig and other mammals in both infants and adults. Cells were described in both a normal state and after the animal had undergone a treatment-inducing blockage in the testicular excurrent ducts. They used these studies to argue that Leydig cells were glandular in nature and whilst they may supply some nutrients to the seminiferous tubules Leydig cells were independent of the seminiferous tubules, and that they appeared to constitute a gland of internal secretion that maintains sexual behaviour and male secondary sexual characteristics. The first 1904 article (Bouin & Ancel 1904) discusses evidence that neither Sertoli cells (cells responsible for nourishment of the sperm during spermatogenesis) or germ cells control male secondary sexual characteristics. The evidence obtained involved; the observation of individuals with cryptorchidism (impaired testicular descent during early development), blockage of the testicular excurrent ducts, compensatory testicular hypertrophy (rapid cell division) after partial castration, and observations on pathological conditions. These were often in combination. Bouin and Ancel found that in animals where the vas deferens (primary sperm transport duct) had been blocked, Leydig cells were more abundant and only Sertoli cells were present in the seminiferous tubules. The second 1904 article (Ancel & Bouin 1904) looked at evidence from embryos, immature animals and old animals, using similar methodology to the first 1904 paper. Leydig cells were abundant in late embryos whilst the seminiferous tubules were underdeveloped at this point, suggesting that Leydig cells control

the development of male characteristics as this is when the formation of the male tract and glands occur.

Bouin and Ancel's 1905 article (Bouin & Ancel 1905) described Leydig cells in the horse over the course of the animal's life stages, from fetus until old age (A fetus of 45 cm, colts at 4, 10, 11, 15, and 25 months of age, and stallions of 3, 8, and 20 years of age), and commented on testis size and Leydig cell population in the testes. The fetal horse testes were found to be surprisingly large (25-40 g per testis), and had a very well developed Leydig cell gland (the fetal interstitial gland). This gland was the main component of the testes as the seminiferous tubules had not fully formed at this stage in fetal development (Fig. 1.3). In colts at approximately 9-10 months, the testes were much smaller compared to the fetal testes due to atrophy of the fetal Leydig cells (FLCs). Leydig cells became well developed again in adult testes and lay between the active seminiferous tubules. In the testes of older animals less Leydig cells were found and testes were much smaller than in younger animals.

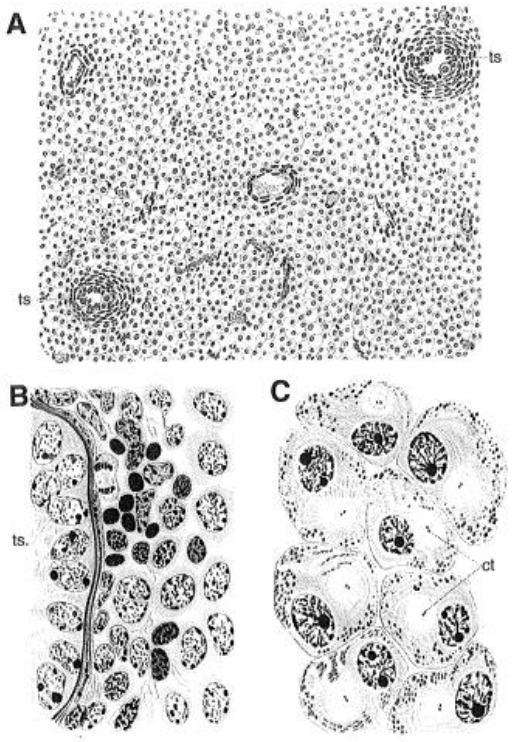

Figure 1.3: First plate from Bouin and Ancel's 1905 article (Bouin & Ancel 1905) looking at testis from a 43 cm horse fetus (A) A low magnification view. Leydig cells fill most of the field. Small seminiferous tubules are seen in the lower left and upper right. (B) A higher magnification showing connective tissue (right) beside a seminiferous tubule (left). (C) Detailed Leydig cells from the same testis as A and B.

Bouin and Ancel's major hypothesis was that the general action of the testes on the individual, which was previously thought to be caused by the testes as a whole, was actually caused by the intersitial gland which was composed primarily of Leydig cells. The four principle pieces of evidence for this were: Firstly, that Leydig cells had the appearance of secretory cells having an epitheliod shape and prominent nucleus. They were also the only cell type in the testis with this morphology. Secondly, secondary male characteristics were preserved in individuals with regressed seminiferous tubules. Despite this regression, Leydig cells were still well developed, even undergoing severe hypertrophy. Thirdly, during embryonic development Leydig cells increase in number during the time when the male reproductive tract was forming; i.e the time at which the seminiferous tubules were

underdeveloped. And finally, Bouin and Ancels 1903 study (Bouin & Ancel 1903) showed that Leydig cells did not primarily function to supply the seminiferous tubules with nutrients.

However, Bouin and Ancel's evidence was all circumstantial and no direct evidence of Leydig cells producing a male hormone would be obtained for another 50 years. This led to many arguments for and against Leydig cells secreting a key male hormone that controlled male secondary sexual characteristics. One key proponent of Bouin and Ancel's hypothesis was Eugen Stienach (1861-1944) who performed a series of studies between 1910 and 1920 (Stienach 1910; Stienach 1911; Stienach 1912; Stienach 1920). His main methodology involved the castration of rats and Guinea pigs followed by the transplantation of the testis to some of the castrated individuals via tissue graft (usually to the inner abdominal wall). Individuals with succesful transplants were compared with castrated individuals with respect to their development of secondary male characteristics. Successful transplants resulted in secondary male characteristics developing with the concurrent development of a large number of Leydig cells in the grafts; indeed they accounted for most of the mass of the grafts. This suggests that the Leydig cells grew rapidly to supply the male hormones that were not currently present in the castrated animals. On the other hand, the seminiferous tubules underwent degeneration. This was indicated by the lack of germ cells present. This provided strong evidence for the hypothesis that Leydig cells were responsible for male secondary sexual characteristics. However, in the 1920s most testicular experts still considered the seminiferous tubules as the most likely source of male hormones. In the 1930s experts opinion began to slowly shift towards the Leydig cell secretion hypothesis. The first direct evidence of this hypothesis was the histochemical identification of 3β-hydroxysteroid dehydrogenase (3β-HSD) a key enzyme in the biosynthesis of steroid hormones (Fig. 1.4). This was shown histochemically using a tetrazolium salt, a dye that is soluble and colourless in its oxidised state but after reduction by 3β-HSD becomes blue and insoluble thus marking

the tissue site of active 3β-HSD (Fig. 1.5). This study was published in 1958 by Lee Wattenberg (Wattenberg 1958) where he used this technique on the adrenal cortex, ovary and testis. On the testis exposure it can be clearly seen that 3β-HSD is found in the intersitial tissue where Leydig cells reside (Fig. 1.6).

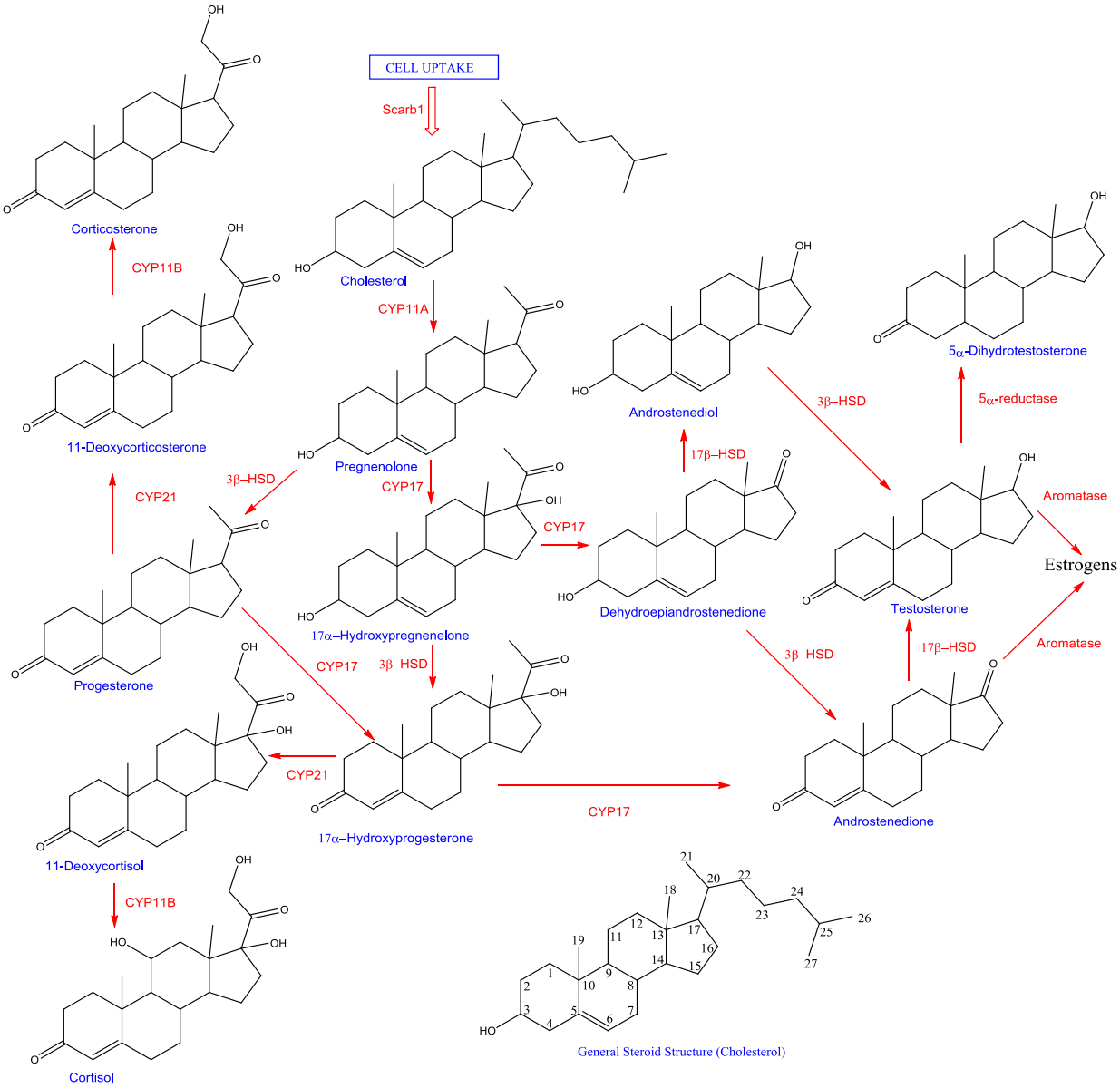

Figure 1.4: Steroidogenesis pathway. Of particular note are the androgens, testosterone and dihydrotestosterone as they are involved in male embryological and pubertal development.

3β-HSD=3β-hydroxysteroid dehydrogenase, 17β-HSD=17β-hydroxysteroid dehydrogenase, Scarb1=Scavenger receptor class B member 1, CYP=Cytochrome P450

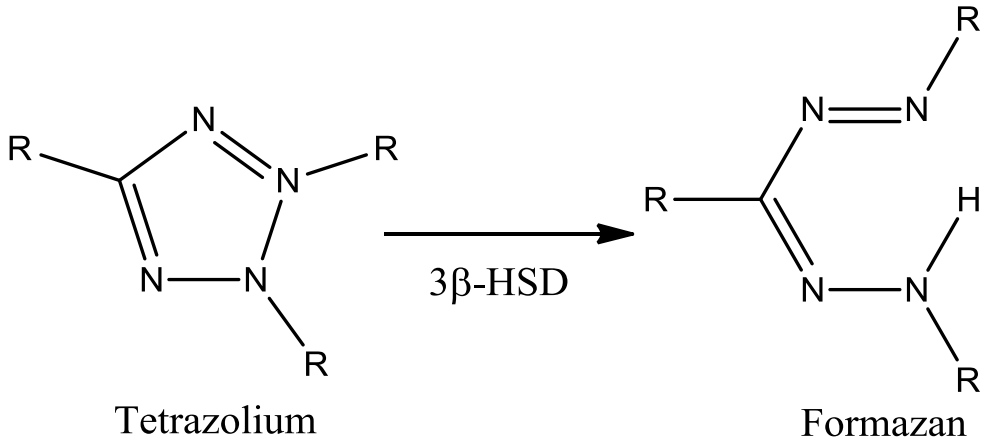

Figure 1.5: Simplified reaction for the reduction of a tetrazolium molecule to a formazan molecule. Tetrazolium salts are soluble and colourless whereas formazan salts are blue and insoluble. One such salt (neotetrazolium) is specific to 3β-HSD. R groups vary between different salts (in neotetrazolium the R groups are benzene rings).

Figure 1.6: Figure showing a section of rabbit testis histochemically stained for the steroidogenic enzyme 3β-HSD from Wattenberg (1958).This assay for 3β-HSD activity (dark stain) was the first direct evidence of Leydig cells as the source of testicular androgens.

To further support that Leydig cells were the site of steroidogenesis in the testis, a biochemical assay was developed in 1965 by Christensen and Mason (Christensen & Mason 1965) which was based on the finding that seminiferous tubules could be separated from the interstitial tissue in rat testes. Both the interstitial tissue (contains Leydig cells) and the seminiferous tubules were cultured separately then exposed to [^{14}C]-progesterone and their [^{14}C]-steroidogenic products were measured. It was shown that the interstitial tissue produced approximately 13.5 times more 17α-hydroxyprogesterone, 7 times more androstenedione, and 3.5 times more testosterone than seminiferous tubules; this proved that Leydig cells were the primary source of androgens in the testes. Later studies have shown that this was the case in other vertebrates (Pudney 1996). These two discoveries definitively proved the theory that the interstitial tissue of testes, mainly composed of Leydig cells, functions as a male hormone-producing gland.

1.1.2. The Origin and Function of Leydig cells

As previously discussed, Leydig cells synthesise a variety of growth factors and hormones, primarily androgens, that facilitate the expression of male traits and behaviours. There are now known to be two Leydig cell populations; FLCs synthesise androgens and growth factors during fetal and embryonic development, and adult Leydig cells (ALCs) synthesise androgens both during and after puberty. Both FLCs and ALCs share similar characteristics such as steroidogenic activity and regulation as well as expressing various markers e.g. the proteins Desert Hedgehog (*Dhh*) and platelet-derived growth factors (PDGF) (both of which are morphogens that induce the expression of early steroid biosynthetic enzymes). Their multiple similarities suggest that they could develop from the same origin and are just different stages of the Leydig cell developmental process. However, after treatment with ethane dimethanesulfonate, which selectively kills Leydig cells (Laskey et al. 1994), ALCs can rapidly repopulate the testis from progenitor cells whilst FLCs cannot (Davidoff et al.

2004). This suggests that FLCs and ALCs could be developmentally distinct steroidogenic populations.

1.1.2.1. Fetal Leydig Cells

FLCs first arise in the mesenchyme (undifferentiated connective tissue) of the developing testis around embryonic day 12.5 (E12.5) in the rat or the 7-8th week of pregnancy in humans (this is shortly after testis cord formation). Their original developmental source remains unclear; however, three potential sources have been proposed. First, they originate from the adrenal-gonadal primordium. This theory is supported by the fact that the adrenal system, particularly the adrenal cortex, can also synthesise androgens and other steroid compounds. This suggests they are of a common origin (Byskov 1986). The transcription factor Steroidogenic Factor 1 (*Sf1)* has been identified in the adrenal-gonadal primordium and continues to be expressed in both the adrenal cortex and gonads once they have separated from one another (Hatano et al. 1996). *Sf1* is essential for the development of steroid producing cells and the expression of steroidogenic enzymes (Ingraham et al. 1994). This finding also suggests that Leydig cells arise from the adrenal-gonadal primordium as *Sf1* is essential for androgen production.

Second, FLCs could originate from the neural crest. The expression of markers normally exclusive to cells derived from neural crest in both fetal and ALCs is evidence of this claim (Davidoff et al. 2002; Davidoff et al. 2004). However, two rat neural crest lineage tracing mouse lines were examined by Brennan et al. (Brennan et al. 2003) and showed no evidence of the neural crest contributing to the FLC population, suggesting that FLCs do not originate from the neural crest.

Finally, FLCs could originate from the coelomic epithelium which encapsulates the gonad. The coelomic epithelium is *Sf1* positive and during testis formation it rapidly proliferates

suggesting it may contribute cells, potentially FLCs, to the testes during its development. Indeed, cells derived from the coelomic epithelium can enter the gonads and differentiate into Sertoli cells (Karl & Capel 1998). This process could also be a source of FLCs.

FLCs could come from one or more of these proposed sources and differentiate in the testis. Once FLCs begin to differentiate they show characteristic features of steroidogenically active cells, one such characteristic is a large smooth endoplasmic reticulum (which is the site of most steroidogenic enzymes) (Kerr & Knell 1988). After their initial differentiation they begin to proliferate rapidly. In rats by E17 there are 2.5×10^4 FLCs per testis and by E21 (immediately before birth) there are approximately 1×10^5 FLCs per testis (A 4-fold increase in numbers) (Kerr & Knell 1988). However, it has been shown that in rats FLC numbers peak around E18 and then begin to steadily decline until the second week after birth (Lording & Kretser 1972). This decrease in FLC number has led to many theories about their developmental fate in adults. Some rat studies suggest that FLCs undergo degeneration (Lording & Kretser 1972; Kuopio & Tapanainen 1989), whilst others suggest that they transform into ALCs (Mendis-Handagama et al. 1998) or persist in the testis along with ALCs (Ariyaratne et al. 2000; Kerr & Knell 1988).

FLCs are essential for three major processes; the development of the Wolffian duct, male external genitalia, and the descent of the testis. The Wolffian duct develops into the epididymis (storage site for newly formed sperm), the vas deferens (transports the sperm for ejaculation) and the seminal vesicles (produces seminal fluid). This process has been shown to be testosterone dependant (Jost 1953). Because Leydig cells are the primary source of androgens, FLCs are essential to this process. Further evidence that androgens are necessary is seen when inactivation of the androgen receptor results in a loss of the Wolffian duct in genetically male rats (Yeh et al. 2002) and humans (Brown 1995). Testosterone is produced by FLCs in the embryological genital tubercle (precursor to the male external genitalia) this is

then converted into 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase (Fig. 1.6) which is expressed in specific tissues. It is now known that DHT is the main factor responsible for developing the male genitalia not testosterone (Russell & Wilson 1994). FLCs also produce insulin-like factor 3 (INSL3) as well as testosterone and both of these are necessary for testes descent. INSL3 promotes transabdominal migration of the testes (Kubota et al. 2001). Testosterone also promotes the formation of the scrotum and is essential for testis descent (Hutson et al. 1997). Inactivation of the testosterone or INSL3 production pathways leads to cryptorchidism (Zimmermann et al. 1999; Adham et al. 2000) which provides further evidence that these factors are essential to this process.

1.1.2.2. Adult Leydig Cells

ALCs go through four main developmental stages; stem Leydig cells (SLCs) (the mesenchymal stem cell population that will eventually differentiate into ALCs), progenitor Leydig cells (PLCs), immature Leydig cells (ILCs), and finally ALCs. SLCs proliferate neonatally and were originally identified because they were present in large numbers in the testicular interstitium prior to ALC formation (Hardy et al. 1989). These stem cells are spindle shaped and do not express steroidogenic enzymes or the luteinizing hormone receptor (LH receptor) which are both characteristic of ALCs (Benton et al. 1995). PLCs arise on postnatal day 11-28 in rats. Like the SLCs they begin spindle shaped and do not express the LH receptor, but unlike SLCs they express steroidogenic enzymes such as 3β-HSD at low activities (Mendis-Handagama & Ariyaratne 2001). Over time, PLCs stop proliferating and enlarge becoming round in shape. They also begin to express more steroidogenic enzymes and at higher activities, but they lack 17β-hydroxysteroid dehydrogenase (17β-HSD) and so accumulate the testosterone precursor androstenedione (Mendis-Handagama & Ariyaratne 2001). PLCs also have high activities of 5α-reductase and 3α-hydroxysteroid dehydrogenase which metabolise androstenedione preventing its secretion at elevated levels with respect to

ALCs and FLCs (Shan et al. 1993). PLCs continue to differentiate forming ILCs the second ALC intermediate. ILCs are most commonly seen in the testes between postnatal days 28-56 in rats and stain heavily for 3β-HSD as well as having high LH receptor activity (all key characteristics of ALCs) (Codesal & Regadera 1990). ILCs are also much rounder than PLCs or SLCs due to their high smooth endoplasmic reticulum content. Another key characteristic that differentiates ILCs from other Leydig cells is their large number of cytoplasmic lipid droplets (Shan & Hardy 1992; Shan et al. 1993). These have a high esterified cholesterol content that can be readily converted to cholesterol, the precursor of steroid synthesis. These lipid droplets diminish when ALCs are formed (Shan & Hardy 1992; Shan et al. 1993). ILCs express 17β-HSD at higher activities by postnatal day 56 completing the androgen biosynthetic pathway and allowing the synthesis of testosterone (Ge & Hardy 1998). The ILCs undergo a final division before full ALC functionality develops on postnatal day 56 (Hardy et al. 1989); cell division and growth come to an end at this stage. ALCs contain a large smooth endoplasmic reticulum (like ILCs), few lipid droplets (suggesting that the primary cholesterol source has shifted from the lipid droplets to cholesterol *de novo* synthesis and low density lipoprotein transport), high levels of steroidogenic activity, and secrete testosterone as their main androgen end product. ALCs also have far less 5α-reductase expression (Ge & Hardy 1998) allowing for production of testosterone without it being further metabolised. This secretion of androgens is essential for pubertal development during the ILC and ALC phases. Because ALCs do not divide, any damage resulting in loss of ALC numbers will be replenished by PLCs and ILCs differentiating into ALCs. However, after puberty PLC and ILC numbers start to decline. ALCs provide a constant level of testosterone during adult life maintaining fertility and male secondary sexual characteristics such as increased bone density.

1.1.3. Steroid Biosynthesis

Steroid hormones have a wide variety of functions; from the stress response associated with cortisol to the sex determining testosterone and 17β-estradiol. The primary tissues that produce steroids are the testes (primarily produces androgens), the ovary (primarily produces estrogens), and the adrenal system (primarily produces corticosteroids). The steroidogenic pathway is catalysed by a range of enzymes and has many intermediates (Fig. 1.6). Each enzyme has a different role in the pathway (even though most enzymes can catalyse multiple steps in the pathway) and each one is tightly regulated by both tissue dependant processes and expression promoting factors. The key steroidogenic enzymes will be outlined below.

1.1.3.1. Steroidogenic Acute Regulatory (StAR) Protein

Steroidogenic acute regulatory (StAR) protein is responsible for the transport of cholesterol, the common substrate for all steroidogenesis, into the inner mitochondrial membrane from the outer mitochondrial membrane. This is the rate determining step of steroidogenesis (Stocco 1997) and because of this StAR is the main regulation point of this pathway. StAR is present in all steroid-producing tissues (including the testes and adrenal system) and is essential for the production of normal levels of steroids. Mutations in the StAR gene result in congenital lipoid adrenal hyperplasia (Lin et al. 1995). This condition is potentially lethal and is characterised by extremely high levels of cholesterol in adrenal and testicular steroidogenic cells. Individuals who have this condition are unable to synthesise normal levels of steroids which results in build-up of cholesterol. The mechanism by which StAR transports cholesterol is unknown, but because StAR is rapidly imported and processed by the mitochondria it has been suggested that contact sites between the inner and outer mitochondrial membrane are formed and cholesterol was transported at these sites (Clark et al. 1994). This mechanism is tenable since similar mechanisms have been observed before

(Schwaiger et al. 1987). However, it is unlikely that this mechanism is essential as removal of 62 amino acids from StAR's N-terminal region, including the mitochondrial binding sequence, does not change its ability to promote steroidogenesis (Arakane & Sugawara 1996). On the other hand, removing the C-terminal region seems to remove StAR's ability to promote steroidogenesis and most mutations are seen in individuals with congenital lipoid adrenal hyperplasia (Miller 1997). Because StAR promotes such a crucial step in steroidogenesis, it is tightly regulated. Regulation of StAR is performed at a *de novo* synthesis level. *Sf1* is an essential promoter for adrenal- and testes-specific expression of steroidogenic cytochromes P450. Steroidogenesis is up-regulated by the chronic stimulation of pituitary peptide hormones, LH in Leydig cells and the ovary, follicle-stimulating hormone (FSH) in the ovary as well as adrenocorticotropic hormone (ACTH) and angiotensin 2 in the adrenal system. The binding of these hormones to their receptors results in an increase in intracellular cyclic adenosine monophosphate (cAMP) concentration. This leads to phosphorylation of promoters of steroidogenic P450 enzymes, resulting in increased expression of StAR, and other steroidogenic enzymes, which leads to more cholesterol transport and consequently enhanced steroid biosynthesis (Gyles et al. 2001; Sugawara et al. 2000).

1.1.3.2. CYP11A *(P450scc)*

The cytochrome P450, CYP11A, also known as cholesterol side chain cleavage enzyme, processes the first catalytic step in steroidogenesis, namely the formation of pregnenolone from cholesterol. It achieves this by hydoxylating two of cholesterols carbon atoms; C22, followed by C20. The C20-22 bond is then cleaved resulting in the products pregnenolone and Isocaproaldehyde (Payne & Hales 2004) (Fig. 1.6). Each oxidation step requires one molecule of O_2 and one molecule of nicotinamide adenine dinucleotide phosphate (NADPH) (Burstein & Gut 1976). CYP11A is coded by a single gene (CYP11A) which has been

isolated from rats (Oonk et al. 1989) and humans (Chung et al. 1986) as well as other species (Venepally & Waterman 1995). CYP11A is located in the inner mitochondrial membrane where the electron transport proteins vital for oxidative catalysis are present. CYP11A is expressed throughout the adrenal cortex as well as Leydig cells, the ovary and the placenta (Oonk et al. 1989; Strauss et al. 1996; Ishimura & Fujita 1997; Payne & Youngblood 1995). Like all steroidogenic P450 enzymes, CYP11A requires the promoter *Sf1* to be present allowing for expression of this enzyme to occur exclusively in steroidogenic tissues. Chronic LH stimulation is needed for optimal expression of CYP11A, like most steroidogenic protiens, CYP11A needs high intracellular cAMP concentration to increase expression, and indeed cAMP responsive regions have been found on CYP11A and CYP17 (Parker & Schimmer 1995; Youngblood & Payne 1992).

1.1.3.3. CYP17 *(P450c17)*

Like CYP11A, CYP17 is a cytochrome P450 enzyme. However, unlike CYP11A, it has two functionalities that can act on two different substrates. The first reaction catalysed is the 17α-hydroxylation of pregnenalone or progesterone to produce 17α-hydroxypregnenalone or 17α-hydroxyprogesterone respectively. The second catalytic activity is cleavage at the C17-20 bond of these products to produce Dehydroepiandrosterone and Androstenedione respectively (Payne & Hales 2004) (Fig. 1.6). This step is essential to synthesise androgens and some corticosteroids. Like CYP11A, one O_2 molecule and one NADPH molecule are necessary for each step (Nakajin et al. 1981). CYP17 is encoded by a single gene (*CYP17*) (Chung et al. 1987). CYP17 is found in the endoplasmic reticulum where, like CYP11A in the mitochondria, the necessary proteins for catalysis are located. CYP17 is expressed in the adrenal gland, Leydig cells, and has limited expression in the ovaries (Endoh & Kristiansen 1996; Sasano & Okamoto 1989; Pelletier et al. 2001). Like CYP11A, CYP17 is regulated by LH and internal cAMP concentration (Youngblood & Payne 1992).

1.1.3.4. CYP19 *(Aromatase)*

CYP19 (aromatase) is also a cytochrome P450 enzyme, it catalyses the conversion of testostereone or androstenedione to estradiol or estrone respectively (Fig. 1.6). This is an essential step in the biosynthesis of estrogens. The mechanism of this reaction involves three catalytic steps; the first two are O_2/NADPH-mediated oxidations of the C19 methyl group, the third step is an O_2/NADPH-mediated elimination of the methyl group producing the steroid product and formic acid (Payne & Hales 2004). A single gene encodes CYP19 (*CYP19*) (Simpson et al. 2002) and it is expressed in the ovary, placenta, and in Leydig cells (Kelch et al. 1972; Kamat et al. 2002; Simpson et al. 2002). Aromatase activity can be stimulated by FSH and human chorionic gonadotropin (pituitary peptide hormones) in rat Leydig cells (Valladares & Payne 1979; Rommerts et al. 1982).

1.1.3.5. CYP21 *(P450c21)*

CYP21 is a cytochrome P450 that catalyses the O_2/NADPH-mediated hydroxylation of the C21 of progesterone or 17α-hydroxyprogesterone producing 11-deoxycortisol and 11-deoxycortcosterone respectively (Fig. 1.6). This is an essential step in corticosteroid synthesis (Payne & Hales 2004). There are two genes, *CYP21A* and *CYP21B*, that encode CYP21; in humans, only CYP21B is active (White et al. 1986). CYP21 is located in the endoplasmic reticulum and is expressed exclusively in the adrenal cortex (Rice et al. 1990). Like other steroidogenic P450 enzymes, CYP21 is up-regulated by chronic stimulation by pituitary peptide hormones; specifically ACTH in the adrenal system (Waterman 1994).

1.1.3.6. CYP11B1

CYP11B1 is a cytochrome P450 that catalyses the 11β-hydroxylation of 11-deoxycorticosterone and 11-deoxycortisol producing the glucocorticoids corticosterone and

cortisol respectively (Fig. 1.6). This reaction is O_2/NADPH-mediated and occurs at the inner mitochondrial membrane where the required electron transport proteins are present (Payne & Hales 2004). CYP11B1 is essential to synthesise corticosteroids. It has two isoforms each encoded by a separate gene. *CYP11B2*, the duplicate gene, has slightly different functionality. These genes are closely linked and are only separated by approximately 40kb in humans (Lifton et al. 1992). Expression of *CYP11B1* is exclusive to the adrenal cortex (Curnow & Tusie-Luna 1991). *CYP11B1* expression is stimulated by angiotensin 2 and by K^+ which increase intracellular Ca^{2+} concentration. Ca^{2+} acts through the same promoter sequences as the intracellular cAMP mechanism (Clyne et al. 1997).

1.1.3.7. 3β-Hydroxysteroid Dehydrogenase (3β-HSD)

3β-HSD is a membrane-bound enzyme that catalyses the conversion of the 3β-hydroxyl group of pregnenalone, 17α-hydroxypregnenalone, androstenediol or dehydroepiandrosterone to produce progesterone, 17α-hydroxyprogesterone, testosterone or androstenedione respectively (Payne & Hales 2004) (Fig. 1.6). This is achieved via two steps; first, the dehydrogenation of the 3β-equatorial hydroxyl group requiring nicotinamide adenine dinucleotide (NAD^+) yielding a 3-keto intermediate and NADH. Second, the oxidation of NADH to isomerise the 3-keto intermediate forming a double bond between C4 and C5 producing the 3-keto-steroid product (Thomas et al. 1995) (Fig. 1.6). 3β-HSD is vital for both androgen and corticosteroid synthesis. 3β-HSD has two isoforms in humans and six in mice, each of these are coded by distinct genes (Morissette & Rheaume 2008; Bain et al. 1993). Human 3β-HSD I is expressed in the placenta, skin, and breast tissue, whereas 3β-HSD II is expressed in the adrenal gland, ovary and testis (Kheaume et al. 1991; Simard & Durocher 1996). This appears to be the same for the mouse 3β-HSD with isoform I being expressed in the adrenal system, ovary, and testes, whereas isoform VI has been observed in skin, placental tissue and testis (Simard & Durocher 1996). Human 3β-HSD II and mouse 3β-HSD

I seem to be regulated by *Sf1* in a similar fashion to the other testis and adrenal specific steroidogenic cytochrome P450s (Leers-Sucheta et al. 1997; Clarke & Bain 1996). However, mouse isoform VI and human isoform I are regulated by two different promoters to regulate placental expression (Peng & Payne 2002).

1.1.3.8. 17β-Hydroxysteroid Dehydrogenase (17β-HSD)

17β-HSD is an essential enzyme in the synthesis of androgens and estrogens. Unlike the other enzymes discussed above, apart from CYP19, 17β-HSD is not involved in glucocorticoid or mineralocorticoid synthesis. It converts the substrates dehydroepiandrosterone, androstenedione and the estrogen estrone to androstenediol, testosterone, and estradiol respectively (Fig. 1.6). It does this by reduction of the 17-keto group utilising NADH. 17β-HSD can be membrane bound or soluble (Payne & Hales 2004). Like 3β-HSD there are multiple isoforms of 17β-HSD; 11 have been identified. However, unlike 3β-HSD, there is little homology between isoforms (Adamski & Jakob 2001; Peltoketo, Luu-The, et al. 1999). Isoforms can differ in substrate specificity, tissue distribution, catalytic preferences (some isoforms can catalyse the reverse reaction with their particular substrate), subcellular localisation and mechanisms of regulation. Each isoform is synthesised by an exclusive gene. Tissue specificity of 17β-HSDs depends on the isoform, e.g. 17β-HSD1 and 17β-HSD7 are both found in the ovary and placenta (these two enzymes synthesise estradiol), whilst 17β-HSD3 is expressed exclusively in the testes (this enzyme irreversibly synthesises testosterone). 17β-HSD1 and 17β-HSD7 are up-regulated by FSH and LH respectively, whilst 17β-HSD3 is up-regulated by gonadotropin stimulation during postnatal development and androgen action after puberty (Peltoketo, Nokelainen, et al. 1999; Baker et al. 1997). Again, control of this enzyme is crucial to cell function and is tightly controlled.

1.1.3.9. Steroid 5α-Reductase (5α-Reductase)

Steroid 5α-reductase (5α-reductase) catalyses the 5α-reduction of the 3-keto C4-C5 double bond in various steroid substrates, either to remove them from the steroidogenesis pathway (e.g. 5α-dihydroprogesterone cannot proceed down the steroidogenesis pathway) or produce vital steroid products. Two such reactions are the conversion of testosterone to DHT or progesterone to 5α-dihydroprogesterone (Fig. 1.6). 5α-Reductase is membrane-bound and uses NADPH as an electron donor. Conversion of testosterone to DHT occurs in most androgen target tissues, including the testes (Ficher & Steinberger 1971). Two isoforms of 5α-reductase have been identified. These two isoforms are coded by distinct genes which have been identified in humans and rats (Russell & Wilson 1994; Mahendroo & Russell 1999). Whether or not a tissue expresses 5α-reductase will determine the primary androgen present in this tissue. If 5α-reductase is not expressed, testosterone (which diffuses across the membrane into the cytoplasm) is the main androgen stimulating male expression. If 5α-reductase is expressed DHT, which is greater than 10 times more potent than testosterone (Deslypere & Young 1992), is the primary androgen stimulant.

1.1.3.10. The Androgen Receptor

As previously stated, testosterone and DHT are the key hormones involved in masculinization both during early development and at puberty. These hormones act through the androgen receptor (AR) (Heemers & Tindall 2007). The AR is expressed in a variety of tissues both reproductive and non-reproductive (Zouboulis et al. 2007). The AR is found primarily in the cytoplasm in its unbound state and is located in a complex with a variety of heat shock proteins and immunophilin chaperones. During androgen binding, the AR undergoes a conformational change and the composition of the protein complex changes allowing the AR to enter the nucleus and form active homodimers (Wilson & French 1976;

Heemers & Tindall 2007). This activated AR complex can then bind to specific DNA sequences known as androgen response elements. The complex will then either directly interact with the transcription preinitiation complex or recruit other proteins that have the capacity to do so (Wang et al. 2005; Heinlein & Chang 2002; Lee & Chang 2003; Heemers & Tindall 2007). This, in turn, induces expression of genes specific for male primary and secondary sexual characteristics. Testosterone, the primary androgen in the blood, and its derivative DHT can both diffuse into a target cell and bind to the AR. If the target cell expresses 5α-reductase, the primary binding androgen will be DHT if it does not express 5α-reductase testosterone will be the primary binding androgen. DHT has a higher affinity for the AR and, consequently, its biological activity is up to 10 times that of testosterone (Deslypere & Young 1992).

1.2. Phthalates and Endocrine Disruption

1.2.1. The Endocrine System

The endocrine system consists of all of the glands responsible for secreting hormones. Along with the nervous system, the endocrine system is responsible for a range of processes all of which are designed to maintain homeostasis. Hormones (unlike neurotransmitters) affect every tissue in the body via their secretion into the blood. There are three major hormone types (based on chemical structure) secreted by the endocrine system; they are as follows:

Firstly, protein and peptide hormones are produced by a wide range of endocrine tissues. These molecules are usually synthesised in an inactive form and then are activated either through the removal of non-functional segments via proteolytic cleavage or via glycosylation of key residues prior to secretion (Smirnova 2000; Leblebicioglu et al. 2013). Protein and peptide hormones are composed of a variety of positive and negatively charged acidic and basic amino acids which result in the hormones overall physiological pH of 7.4. Because

water can interact with these charged amino acids protein and peptide hormones are hydrophilic and are easily transportable to tissues via the blood. However, this hydrophilicity makes it difficult for them to pass through the cell membrane. Because of this, the majority of the receptors associated with protein and peptide hormones are membrane bound and consist of an extracellular binding domain for the hormone and an intracellular domain which will cause an intracellular response after the hormone is bound (Smirnova 2000; Leblebicioglu et al. 2013). Due to their hydrophilicity protein and peptide hormones can easily be excreted without any modification. However, there are enzymes that will inactivate protein and peptide hormones to allow for the tight control of signal duration (Glass et al. 1969). Examples of protein and peptide hormones are: LH, FSH, adrenalcorticotropic hormone (these three stimulate steroid synthesis in different tissues), glucagon, and insulin (these two regulate blood glucose levels) to name a few (Leblebicioglu et al. 2013).

Secondly, there are hormones that are derived from a single amino acid. Dopamine and epinephrine are amine hormones that are both derived from tyrosine (Kopin 1968) though amine hormones can also be derived from other amino acids. Amine hormones, like protein and peptide hormones, are hydrophilic and are freely soluble in the plasma. This means that, like protein and peptide hormones, amine hormones bind to the extracellular regions of membrane bound receptors. Amine hormones are broken down by monoamine oxidases to form hormonally inactive compounds (Kalgutkar et al. 2001). The thyroid hormones (thyroxine and triiodothyronine), like dopamine and epinephrine, are synthesised from tyrosine (Kinne et al. 2011). However, because they contain two nonpolar iodinated rings they are hydrophobic (Piehl et al. 2011). Because of this they do not dissolve in the plasma and thus they are transported on plasma carrier proteins. On the other hand, they can also either diffuse through the cell membrane or be transported through the membrane by transport proteins allowing them to interact with both surface receptors and intracellular

receptors (Leblebicioglu et al. 2013; Piehl et al. 2011). Thyroid hormones are firstly deiodinated and then are conjugated via glucuronidation or sulphation prior to excretion.

Lastly, there are the steroid hormones which are all synthesised from the common precursor cholesterol (Gimpl et al. 2002). Because cholesterol is such a nonpolar, hydrophobic molecule steroids are also poorly soluble in water. Due to this hydrophobicity, like the thyroid hormones, steroids are reversibly bound to carrier proteins that transport them to their target tissues. Steroids can also bind to both extracellular receptors (such as estrogen receptor α) or move through the cell membrane via either diffusion or transport proteins allowing them to interact with intracellular receptors (such as the AR) (Nemere et al. 2003). Steroid hormones are conjugated by phase 1 and 2 metabolism producing hydrophilic glucuronide and sulphate conjugates allowing them to be excreted rapidly in water-based excretory fluid (e.g. urine). Steroids have a variety of functions some examples include: 17β-estradiol which is the primary estrogen responsible for female development (Simpson et al. 2002); testosterone and DHT which are the primary androgens responsible for male development (see section 1.2.2.1); cortisol which is involved in the stress response; and aldosterone which promotes Na^+ uptake.

Hormone production itself is also tightly regulated via a combination of feedback mechanisms to allow control of the synthesis, secretion and degradation of hormones (Fig. 1.7). Hormones are also rapidly degraded to make sure hormonal signals have a very limited time scale. This is vital because hormones act at very low concentrations and thus a small increase or decrease in hormone activity will produce a significant biological effect. However, there are multiple chemicals that disrupt the synthesis, secretion, transport, binding, action, or degradation of hormones. A chemical that does this is known as an endocrine-disrupting chemical (EDC).

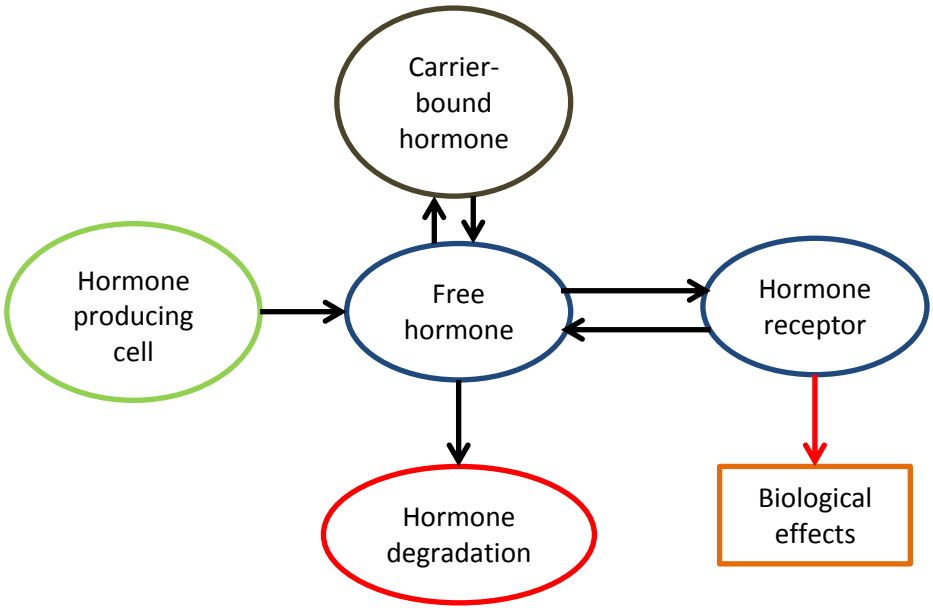

Figure 1.7: The generalised production, activity and degradation of a hormone. Note that only hydrophobic hormones will be bound to carriers. Hormone production and degradation are tightly controlled to ensure that the appropriate levels of hormone are in circulation (both in bound and unbound forms).

1.2.2. Endocrine-Disrupting Chemicals and Reproductive Health

An endocrine disruptor has been defined as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (WHO & UNEP 2013), this includes EDCs. EDCs can interfere with hormones in a number of ways, including hormone production, release, metabolism, and degradation as well as being able to mimic the hormones and interact with their receptors (Tabb & Blumberg 2006). EDCs could potentially target a whole range of metabolic processes (it has even been theorised that EDCs metabolic interference could contribute to the growing obesity rate (Baillie-Hamilton 2002)). However, due to multiple observations showing an increased incidence of hyperthyroidism in cats from 1 in 1000 in the 1970s-80s to 21 in 1000 in the 1990s (Edinboro et al. 2004) (making hyperthyroidism the most common endocrine disease in small animal diagnoses) EDCs targeting the thyroid response are being investigated (Boas et al. 2006). Also, because

multiple reports have shown an increase in the number of reproductive disorders (such as the increased female infertility rate and decreased sperm count) in both adults and during embryonic development (for example the increased incidence of male developmental defects such as cryptorchidism (failed descent of the testes) and hypospadias (malformation of the urethra)) most EDC studies have focussed on the sex steroid hormones (Crain et al. 2008; Sharpe & Skakkebaek 2003).

EDCs that influence the production, transportation and activity of the sex steroids (specifically 17β-estradiol and testosterone) can originate from a variety of sources including pesticides, plastics, fungicides, industrial by-products, and some are even synthesised by biological organisms (Cimafranca et al. 2004; Grün et al. 2006; Hoppe & Carey 2007; Akingbemi et al. 2007; Lee et al. 2008; Hines et al. 2009; Park et al. 2010; Casals-Casas & Desvergne 2011). Because of their widespread use/production EDCs are ubiquitous environmental contaminants. These compounds can act via three primary modes of action; mimicking the hormone and binding to the receptor providing a reduced hormone response (i.e. a receptor agonist), mimicking the steroid and binding to the receptor resulting in enzyme inhibition. Thus, making it unable to be accessed by the target hormone (i.e. a receptor antagonist), or altering steroid production by influencing the expression or activity of key enzymes in the steroidogenesis pathway (Fig. 1.8).

A commonly observed mode of EDC action is binding of the EDC to a hormone receptor inducing the same response as the target hormone. A common class of EDCs that utilise this mechanism are the xenoestrogens which contain a number of compounds that bind to the estrogen receptors (ERs) ERα and ERβ (Casals-Casas & Desvergne 2011). Xenoestrogens can originate from natural sources such as the plant-produced phytoesterogens. We are constantly exposed to phytoestrogens through our diet. For example, the phytoestrogen genistein produced by soy. Anyone with a soy component in their diet will be regularly

exposed to genistein which will bind to the ERs and induce an estrogenic response (Kurzer & Xu 1997). However, these natural compounds have existed for as long as the plants they're derived from. The much more recent synthetic xenoestrogens have been the main cause for concern. Prime examples of synthestic xenoestrogens are plasticisers and monomers of polycarbonate plastics such as bisphenol A (BPA) which is commonly used as both. BPA is commonly found as a polymer in polycarbonate containers that are used to store food and drink. BPA can bleed into the food/drink and upon consumption will cause an estrogenic response. Studies have shown that one week of polycarbonate bottle use increases urinary BPA content by two thirds (Carwile et al. 2009) and BPA is known to cause estrogenic effects in humans such as precoucius puberty in girls (Howdeshell & Hotchkiss 1999; Krishnan & Stathis 1993). Xenoestrogens bind to one or both of the ERs causing unregulated expression of female traits. Drinking from cans prior to the removal of plastic internal lining has shown to cause a significant increase in BPA exposure (Matsumoto et al. 2002).

Another method of EDCs' activity is similar to the first in that the EDCs still bind to the hormone receptor but instead of mimicking the hormone's response these compounds inhibit the receptor occupying its binding site preventing the hormone from activating the receptor. An example of an EDC that act via this mechanism is the anti-androgen fungicide vinclozolin. Vinclozolin is a commonly used fungicide in vineyards as well as fruit and vegetable plantations so we are regularly exposed via diet. However, the exposure levels the general population are exposed to are not considered to be significant. Farm workers on the other hand, are exposed to levels which could produce adverse effects (WPPR 1998). Vinclozolin has been shown to cause male developmental defects, such as hypospadias, by inhibition of AR-mediated gene activation (Wong et al. 1995; Kelce et al. 1994). Other anti-androgens with this activity are the herbicide linuron and the fungicide procymidone

(Svechnikov et al. 2010). These compounds also cause increased incidences of reproductive defects such as hypospadias and cryptorchidism in animals (Gray et al. 2001).

A third mechanism of EDCs action is by disrupting the biosynthesis of hormones by interfering with key enzymes in their biosynthetic pathways. For example, some xenoestrogens can interfere with testosterone synthesis. Dichlorodiphenyldichloroethylene (DDE), a stable metabolite of the ubiquitous environmental contaminant dichlorodiphenyltrichloroethane (DDT); DDT's use is now restricted due to its long biodegradation time; has been shown to stimulate aromatase activity and act synergistically with FSH (a pituitary peptide hormone) to stimulate StAR and early pathway CYP activity in human granulosa cells (steroid producing cells located in the ovary) (Younglai et al. 2004; Whitehead & Rice 2006). Lindane, a pesticide that is still used, inhibits progesterone production in a dose dependant manner in mouse MA-10 cells (an immortalised mouse Leydig cell tumour line that can produce early steroids) (Walsh & Stocco 2000). It does this by decreasing internal cAMP stimulation of StAR thus preventing the movement of cholesterol to the inner mitochondrial membrane where the enzyme responsible for progesterone synthesis (CYP11A) is located.

Any of the mechanisms of EDCs action discussed above would result in an overall change in hormone activity (Fig. 1.8) and there are myriad EDCs that act on estrogen and androgen hormonal activity via these mechanisms. However, many EDCs use a combination of these methods to achieve the reproductive disorders seen in exposure studies. For example, DDE does not just upregulate StAR, CYP, and aromatase activity resulting in more free estrogens; it also acts as an anti-androgen and binds to the AR (Kelce et al. 1995). These two activities both reduce testosterone activity and increase estrogen activity and because activated ERα supresses Leydig cell testosterone production (Akingbemi 2003; Kalla et al. 1980) testosterone activity is further reduced. BPA also acts in a similar manner as it has been

shown to both upregulate aromatase (Nativelle-Serpentini et al. 2003) and antagonise the AR (Sohoni & Sumpter 1998). Because humans are exposed to multiple EDCs the combination of several of these compounds should be taken into consideration when discussing acceptable doses. Because multiple EDCs act via the same mechanism (e.g. xenoestrogens all act as estrogen receptor agonists) most combinations will have at least an additive effect. Some xenoestrogens even act synergistically on the ERs causing a higher estrogenic response than their individual responses combined (Arnold et al. 1997). On top of this, it is possible for EDCs to have a multigenerational effect. BPA is known to have an epigenetic effect via alterations of DNA methylation patterns resulting in changed gene expression over multiple generations (Dolinoy 2008). *In utero* exposure to the effects of these compounds could have an even greater impact on reproductive development. Increased exposure to synthetic EDCs explains the gradual increase in the incidence of reproductive defects such as decreased sperm motility, genital defects, and infertility due to their effects on estrogen and androgen activity. Therefore, it is important to understand the mechanisms underlying the action of these EDCs so appropriate regulatory steps can be put in place to decrease exposure to levels where no adverse effects occur.

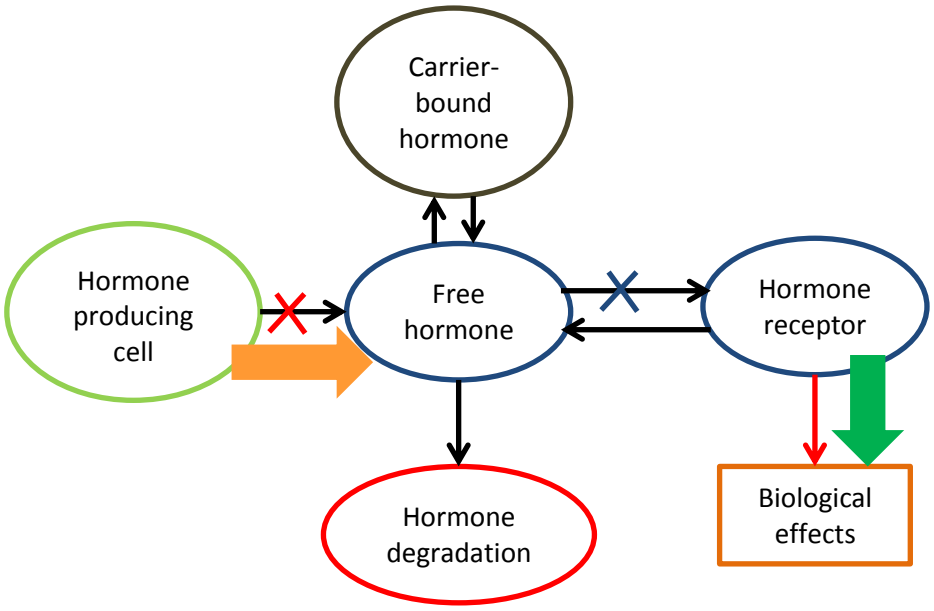

Figure 1.8: EDCs potential methods of action on hormone activity. ► = Hormone mimics amplify the biological effects by binding to the hormone receptor. X = Receptor inhibitor prevents hormone binding decreasing biological effects. X = Synthesis inhibitors prevent biosynthesis of hormones meaning a lower free hormone concentration resulting in less receptor binding and less biological effects. ► = Synthesis promoter leading to increased free hormone concentration and increased receptor binding resulting in amplified biological effects.

1.2.3. The Toxicity of Phthalates

Phthalate diesters (phthalates) are a class of chemical that are commonly used as plasticisers and pesticides in a variety of products such as children's plastic toys, food packaging, cosmetics, medical equipment (including surgical equipment), acaricides, and are also used as solvents for inks (Foster et al. 2000; Xiao-feng et al. 2009; Janjua et al. 2007; Carran & Shaw 2012; Svechnikov et al. 2010). Phthalates are composed of a benzene ring with two esters groups ortho to each other, making them phthalic acid molecules with ester linkages instead of carboxylic acid moieties (Fig. 1.9). The two phthalates most commonly used are diethylhexylphthalate (DEHP) and dibutylphthalate (DBP).

Because of their widespread use phthalates are ubiquitous environmental contaminants (Fromme et al. 2002) meaning that humans and wildlife are regularly exposed to multiple

phthalates on a daily basis. It has been estimated that the average adult in Denmark is exposed to DBP doses between 1.8-4.1 μg/kg body weight/day and that children between 1-6 years old are exposed to 8 μg/kg body weight/day through their diet (EFSA 2005). There are also groups that are exposed to higher phthalate levels through occupational exposure. For example, Chinese construction workers that regularly work with DBP and DEHP had significantly higher levels of phthalate metabolites in their urine (DEHP metabolites are 100x more concentrated) when compared with construction workers that do not work with DBP and DEHP (Pan et al. 2006). Phthalates are known EDCs (Mankidy et al. 2013; Pant et al. 2011) and are associated with male genital developmental disorders such as hypospadias and cryptorchidism in a wide range of animal species including frogs (*Rana rugosa*) (Ohtani et al. 2000) and rats (Wolf et al. 1999; Parks et al. 2000). Exposure of pregnant rats to phthalates resulted in a host of developmental defects including cryptorchidism, hypospadias, and testicular cancer. For example, Mylchreest et al (1998) found that increased DBP exposure to pregnant dams induced male genital malformations and reduced anogenital distance (AGD) (a small AGD is associated with an increased incidence of cryptorchidism) in their offspring in a dose dependant manner. A similar study by Gray et al (2000) showed that multiple other phthalates also induced this effect (e.g. doses of 750mg/kg body weight/day of DEHP in pregnant rats induced a 82% male genital malformation rate in their offspring).

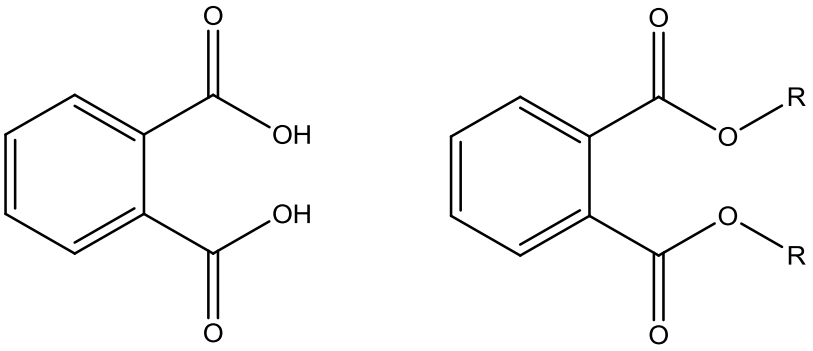

Figure 1.9: The general structure of a phthalate and the structure of phthalic acid. Both have a benzene ring with two side chains ortho of each other. In phthalic acid these side chains are carboxylic acid moieties. In phthalates these are ester linkages. The R group attached to these ester linkages defines which specific phthalate the molecule is e.g.
Dibutylphthalate (DBP); R = $CH_2CH_2CH_2CH_3$
Diethylhexylphthalate (DEHP); R = $CH_2CH(CH_2)_2CH_2CH_2CH_2CH_3$

Phthalates are known to cause these reproductive defects by decreasing testosterone synthesis in a dose additive manner (Howdeshell et al. 2007; Howdeshell et al. 2008; Mylchreest et al. 2002; Parks et al. 2000) as well as decreasing the expression of INSL3 (a key molecule needed for testes descent) in FLCs (resulting in decreased mRNA levels) (Wilson et al. 2004). Because testosterone and INSL3 are key molecules in early male genital development this explains the defects seen in exposed rats. This means that phthalate's endocrine-disrupting activity is achieved by disrupting hormone synthesis rather than interacting directly with the AR. The fact that phthalates toxicities are additive suggests that they act via a common mechanism of toxicity. This mechanism is not yet fully understood.

When phthalates are absorbed into an animal they are rapidly metabolised by esterases forming a monoester metabolite (e.g. DBP is metabolised to monobutylphthalate (MBP)) (Fig. 1.10). These esterases are primarily located in the liver (Hanioka et al. 2012) but non-specific esterases found throughout the body (including the Leydig cells) can also catalyse this process (Molenaar et al. 1986). MBP can then be further hydrolysed by esterases to form phthalic acid or it can be hydroxlated and/or glucuronidated by phase 1 and phase 2

metabolism and excreted (Fig. 1.10). The first step in the DBPs *in vivo* metabolic pathway occurs very rapidly. In rats dosed with 100 mg/kg [^{14}C]-DBP no DBP was detected in the plasma or urine. MBP and MBP glucuronide however were the two major DBP metabolites found in both urine and serum within two hours of administration (Fennell et al. 2004). Because phthalate monoesters are the main active metabolites *in vivo*, it is considered that they are the molecules that caused their respective phthalate's endocrine-disrupting activity. To support this, multiple phthalate monoesters, including MBP and monoethylhexylphthalate (MEHP) (DEHP's monoester metabolite), have been shown to decrease testosterone production in a dose dependant manner in MA-10 cells (Clewell et al. 2010).

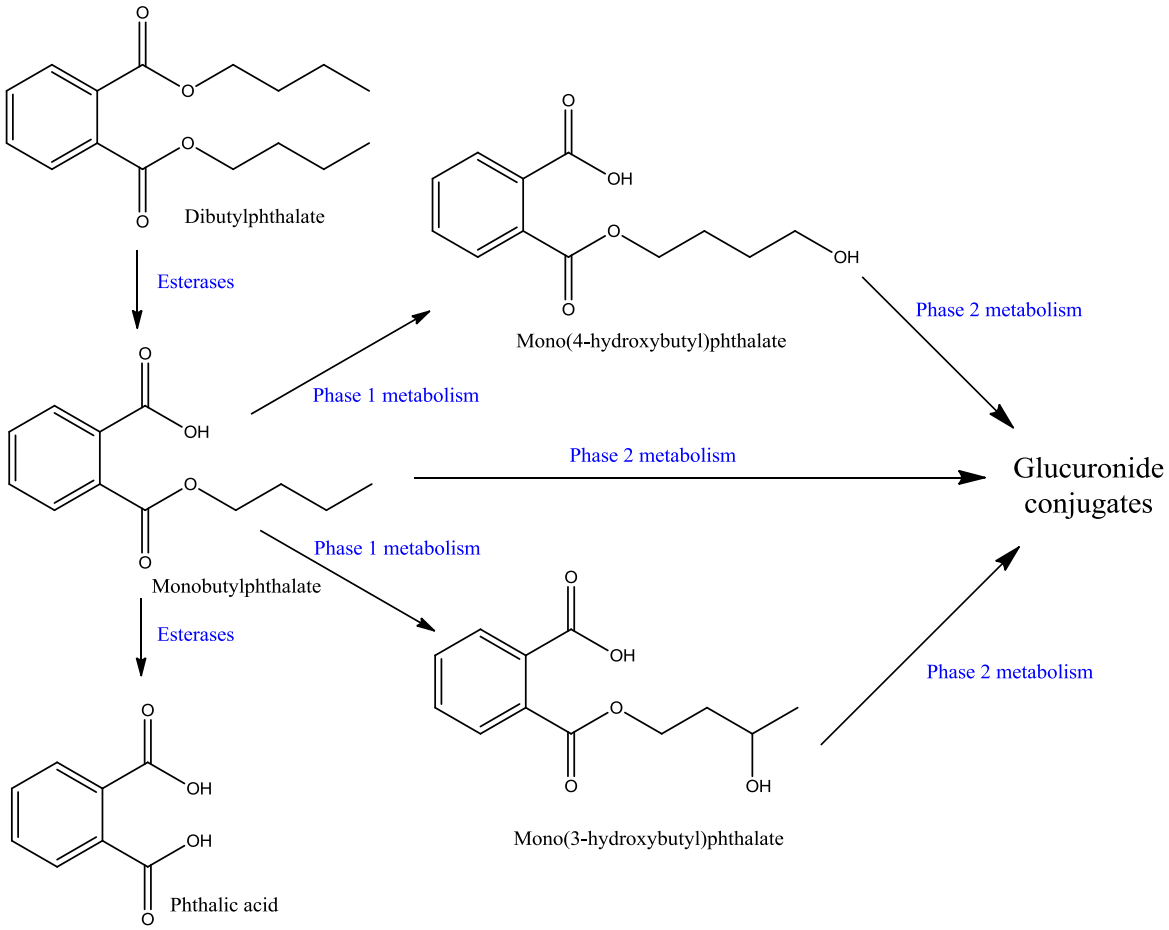

Figure 1.10: The metabolism of DBP in mammals. The formation of MBP and phthalic acid is catalysed by esterases. MBP can also enter phase 1 metabolism to produced hydroxylated products these will then be glucuronidated by phase 2 metabolism along with MBP.

Several theories have been proposed for phthalates mechanism of toxicity. One such mechanism is the direct inhibition of key steroidogenic enzymes. Yuan et al (2012) tested this by isolating human and rat 3β-HSD and 17β-HSD and ran a kinetic analysis of these enzymes in response to various phthalates and their monoester metabolites. They found that whilst some phthalates caused competitive inhibition with respect to the steroid precursor the monoesters did not have any significant effect on catalysis (Yuan et al. 2012).

A recent study suggested that phthalates' effects are mediated by increasing the levels of reactive oxygen species (ROS) Leydig cells are exposed to. They reported that at low doses MEHP stimulated steroidogenesis and increased the expression of StAR and other key steroidogenic enzymes. But, at high MEHP doses the oxidative stress caused by the increased presence of ROS caused a decrease in the expression of steroidogenic enzymes (StAR, CYP11A, 3β-HSD, and 17β-HSD) and therefore caused a decrease in testosterone synthesis (Zhao et al. 2012).

Another proposed mechanism of phthalate toxicity is via the suppression of early steroidogenic enzyme expression. Clewell et al (2010) determined that both phthalate diesters and monoesters inhibited LH stimulation in mouse MA-10 Leydig cells. This resulted in decreased expression of a number of key steroidogenic enzymes including StAR, CYP11A, and CYP17 leading to decreased testosterone synthesis (Clewell et al. 2010). Other studies have also shown decreases in steroidogenic enzymes including 3β-HSD, StAR and CYP17 in the testis of rats exposed *in utero* (Lehmann et al. 2004). However, another study suggests that phthalates (specifically DBP) caused an increase in serum glucocorticoids (Xiao-feng et al. 2009); glucocorticoids would then bind to the glucocorticoid receptor causing the same down-regulation as seen in the Clewell et al (2010) and Lehmann et al (2004) studies.

On top of these proposed mechanisms recent studies have shown that phthalates (specifically DBP) may have a multigenerational effect. The children of New Zealand male soldiers exposed to high levels of DBP used as an acaricide (to kill Trombiculid mites that carry bush typhus) had a higher incidence of the male genital defects, cryptorchidism and hypospadias than the general population (Carran & Shaw 2012; Carran & Shaw 2010). The incidences reported by Carran and Shaw (2012) have been disputed, but it is accepted that there is a significant difference between the exposed soldier's children and the general population (Elwood & Borman 2012). This suggests that the toxicity of phthalates might be inherited due to an epigenetic effect that could be passed to the next generation via sperm. This has been seen in BPA's mechanism of toxicity which acts by altering DNA methylation patterns (i.e. epigenetically); therefore, an effect such as this could be possible. However, the majority of studies look at the exposure of pregnant rats and so reflect *in utero* exposure and not multigenerational effects. The closest to studying the epigenetic effects produced by phthalates was the exposure of rats to a combination of DBP, DEHP, and BPA, an increased incidence of testicular disease was seen in both the F1 and F3 generations (Manikkam et al. 2013). However, whether phthalates contributed to this multigenerational effect is unknown. In order to further understand the biochemical mechanisms of phthalates' effects on male development, an understanding of the impact of phthalates on steroidogenesis in Leydig cells is necessary.

1.3. Research Objectives

The aim of this research is to investigate the effects of DBP on the steroidogenesis pathway in the cultured rat Leydig cell cancer line R2C.

This aim will be achieved through the following research objectives:

1. To develop, and characterise the R2C and LC-540 Leydig cell culture systems and their steroidogenic biochemistry.
2. To develop an analytical protocol that can both detect and quantify selected steroids secreted by cell cultures.
3. To collect, analyse, and assess any changes in steroid levels produced by R2C cells exposed to varying concentrations of DBP using the developed analytical method.

Chapter 2 - Materials and Methods

2.1. Materials

2.1.1. Chemicals

All chemicals used were of general laboratory grade except:

- Dibutyl phthalate (Sigma-Aldrich New Zealand Ltd, Manukau City, New Zealand)
- Benzyl penicillin (Sigma-Aldrich New Zealand Ltd)
- Streptomycin sulphate (Sigma-Aldrich New Zealand Ltd)
- Ground dextran coated charcoal (Sigma-Aldrich New Zealand Ltd)
- Trypan blue (Sigma-Aldrich New Zealand Ltd)
- High performance liquid chromatography (HPLC) grade Acetonitrile (Acetonitrile gradient grade for liquid chromatography, Merck Millipore, Manukau City, New Zealand)
- Analytical grade methanol (ECP ltd, Auckland, New Zealand)
- Analytical grade diethylether (ECP ltd)
- Synthesis grade trifluoroacetic acid (Scharlau, Barcelona, Spain)
- MilliQ water was prepared using Q-Guard 1 purification pack (Merck Millipore) the water used had a resistance of 18.2 MΩ/cm.

2.1.2. Biological products

- Phenol red-free MEM powder (Sigma-Aldrich New Zealand Ltd)
- Phosphate buffered saline (Sigma-Aldrich New Zealand Ltd)
- Fetal bovine serum (Life Technologies, Auckland, New Zealand; Catalogue number 10091-148)
- Eagle's Minimum Essential Medium (Life Technologies)
- TrypLE express (Life Technologies)

- Trypsin powder (Becton Dickinson, Auckland, New Zealand)
- R2C rat Leydig cells (American Type Culture Collection (ATCC), Manassas, USA; ATCC number CCL-97)
- LC-540 rat Leydig cells (American Type Culture Collection (ATCC); ATCC number CCL-43)

2.1.3. Equipment

- T-75 sterile culture flask (Sigma-Aldrich New Zealand Ltd)
- Sterile filter (Steritop-GP, 0.22 µm, polyethersulfone, 500 mL 45 mm Merck Millipore)
- Laminar flow cabinet (Cytoguard CG2000 series, model CGA-180, Clyde Apac, Sydney, Australia)
- Inverted microscope (CKX41, Olympus, Melbourne, Australia)
- Non sterile syringe filter (7.5mm non-sterile PTFE hydrophobic filter, Thermo Fisher Scientific, Melbourne, Australia)
- Dionex brand HPLC system (P680 HPLC pump, automated sample injector ASI-100, thermostated column compartment TCC-100, Thermo Fisher Scientific)
- Centrifuge (multifuge 1 S-R, Heraeus, Hanau, Germany)

2.2. Cell Culture

Cell culture procedures were developed in collaboration with my MSc student colleague Kristie Webber. This section represents joint work.

2.2.1. Culture Media Preparation

2.2.1.1. Heat Inactivation of Fetal Bovine Serum (FBS)

The heat inactivation procedure developed by the Alliance for Cellular Signalling (Davis & Hsueh 2002) was used as follows:

A 500mL bottle of fetal bovine serum (FBS) was taken from storage at -20 ℃ and thawed overnight at 4 ℃, followed by gentle warming in a 37 ℃ water bath making sure the water level was above the serum level in the bottle. The serum was gently mixed by inversion to ensure a uniform temperature throughout. Following thawing the serum was incubated for an additional 15min to achieve a final serum temperature of 37 ℃. The temperature of the water bath was raised to 56 ℃ and the serum was allowed to warm to 56 ℃ over 35 min. During this time the serum was gently inverted every 10 min. When the serum temperature was at 56 ℃ it was incubated for a further 30min and the bottle was gently inverted every 10 min. The serum was then removed and cooled to room temp (30min). Serum (100 mL for 10% medium, 175 mL for 17.5% medium) was transferred to 200 mL sterile Schott bottles and stored at -20 ℃.

2.2.1.2. Preparation of Phosphate Buffered Saline (PBS)

MilliQ water (900 mL) was added to a 1L Schott bottle. One bottle of phosphate buffered saline (PBS) powder (9.6 g) was added to the PBS in the Schott bottle with continuous swirling of the liquid. A small amount (approx. 5 mL) of milliQ water was used to rinse any remaining PBS powder out of its original bottle and added to the Schott bottle. The pH of the PBS was adjusted to 7.4 using 1M HCL or 1 M NaOH. The resulting solution was made up to 1 L with MilliQ water. The PBS was redistributed into 100-200 mL aliquots and autoclaved (see section 2.2.1.5.1.). After autoclaving it was stored at 4 ℃.

2.2.1.3. Serum Stripping Process

A solution containing 0.3 g/L $MgCl_2(H_2O)_6$, 85.6 g/L sucrose, 2.4 g/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) in 1 L MilliQ water was divided (50 mL aliquots) into 50 mL plastic centrifuge tubes. Ground dextran coated charcoal (127 mg) was added to each and the centrifuge tube aliquots were inverted 10 times to allow even distribution of the charcoal. They were incubated at 4℃ for 24 hours. The solution was centrifuged at 500 xg for 10 min and the supernatant was poured off and 50 mL of inactivated FBS was added to the charcoal pellet in each centrifuge tube. The tubes were inverted 10 times and incubated for 24 hours at 4℃. The solution was centrifuged at 1700 xg for 10 min and the stripped FBS was decanted into 200 mL Schott bottles in aliquots (100 mL for 10% medium, 175 mL for 17.5% medium) and stored at -20℃ (Blom et al. 1998).

2.2.1.4. Preparation of Penicillin and Streptomycin Solutions

Benzyl penicillin (3 g) and streptomycin sulphate (2.8 g) was added to 100 mL MilliQ water and stirred overnight (magnetic stirrer) to completely dissolve the antibiotics. The solution was filtered into 5 mL aliquots using a sterile filter. The aliquots were stored at -4℃.

2.2.1.5. Sterilisation of Glassware

2.2.1.5.1. Autoclaving

Glassware and pipette tips were autoclaved at 121℃, 15 psi for 20mins.

2.2.1.5.2. Use of 70% v/v Ethanol (aq) Spray

Glassware and metal instruments were sterilised by spraying with an aerosol of 70% (v/v) ethanol (aq) before transferring to a laminar flow cabinet.

2.2.1.5.3. Maintaining Sterility within the Laminar Flow Cabinet

All sterile procedures were carried out in a laminar flow cabinet. The internal surfaces of the cabinet were pre-sterilised by exposure to germicidal UV light (254 nm for 2 hours). Immediately prior to use they were sprayed with 70% v/v ethanol (aq) aerosol.

2.2.1.6. Preparation of Eagle's Minimum Essential Medium (MEM) Containing 17.5% FBS

Heat inactivated FBS (see section 2.2.1.1.) (175 mL, stored at -20℃) was defrosted at 37℃ in a water bath (15-20 min). Two 500 mL bottles of MEM, two 5 mL vials of pre-prepared antibiotics (see section 2.2.1.4.), a 1 L sterile Schott bottle, a sterile filter in a sealed bag, and defrosted FBS were sterilised (see section 2.2.1.5.). The filter system was attached to the empty sterile Schott bottle and a vacuum was used to pull the liquid into the sterile bottle (Fig. 2.1). The bottle and cap were flame sterilised. The bottle of medium was removed from the laminar flow cabinet, labelled and stored at -4^{o}C.

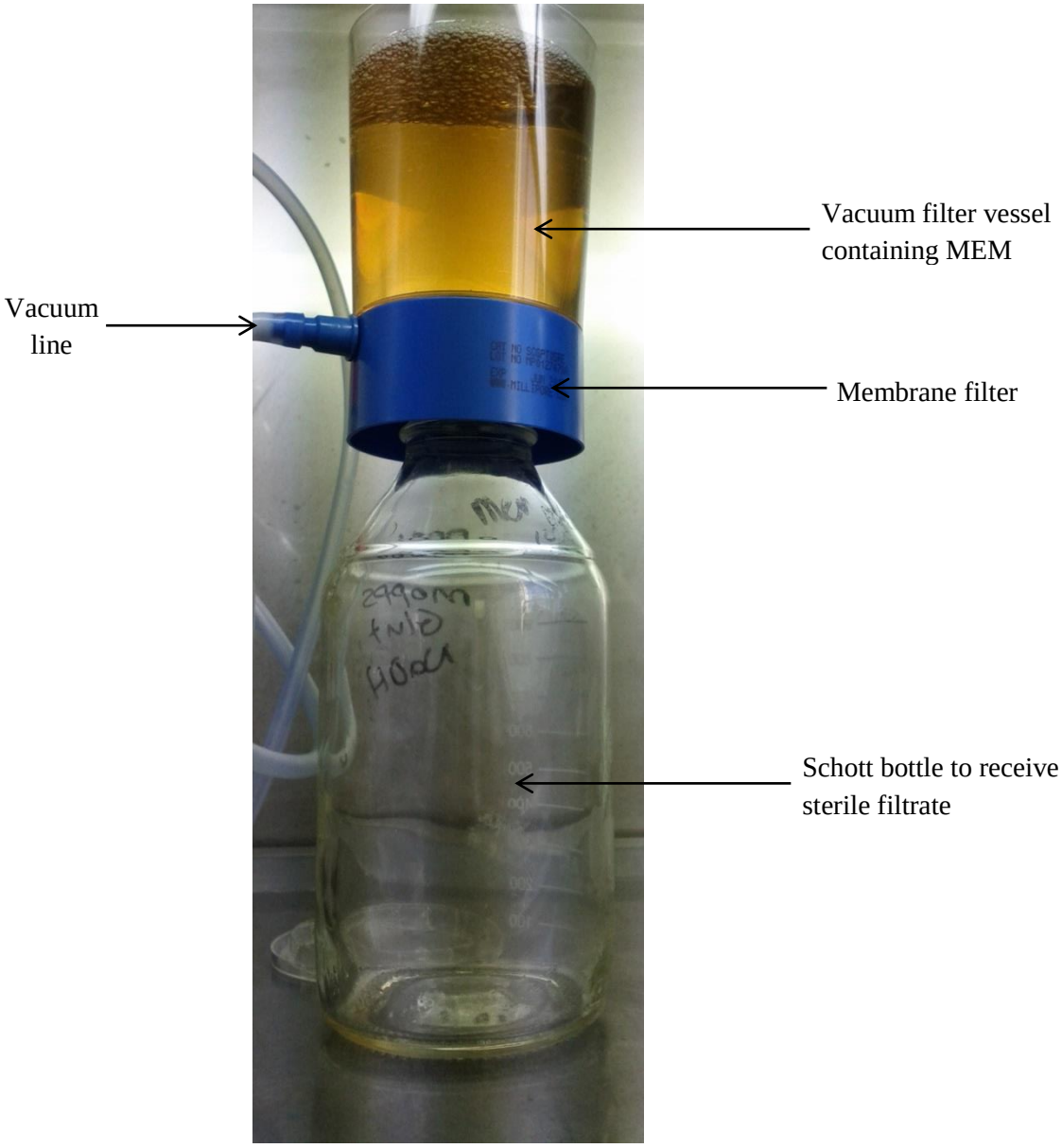

Figure 2.1: Vacuum filter containing 500 mL of MEM attached to an empty Schott bottle.

2.2.1.7. Preparation of Phenol Red-Free MEM Containing 17.5% Stripped FBS

Frozen stored stripped FBS (175 mL) was defrosted (15-20 min) in a 37 ℃ water bath. One bottle of phenol red-free MEM powder was added to 825 mL of milliQ water in a 1 L Schott bottle whilst gently swirling to dissolve. The phenol red-free MEM powder bottle was rinsed with a small amount (approx. 5 mL) of milliQ water to remove any remaining powder; this was added to the Schott bottle. Sodium hydrogen carbonate (2.2 g) was added to the solution

in the Schott bottle, this was swirled until dissolved. The solution was then adjusted to pH 7.4. The phenol red-free MEM was sterilised by filtration (see section 2.2.1.6.) and stored at 4℃

2.2.1.8. Preparation of Eagle's Minimum Essential Medium (MEM) Containing 10% FBS

The procedure was the same as that described in section 2.2.1.6. except 100 mL of inactivated FBS was used.

2.2.1.9. Preparation of Phenol Red-Free MEM Containing 10% Stripped FBS

The procedure was the same as that described in section 2.2.1.7. except 100 mL of stripped FBS and 900 mL of milliQ water were used.

2.2.1.10. Trypsin Preparation

NaCl (8.5 g) was dissolved in 1 L of MilliQ water, trypsin powder (25 g) was added to the 0.85% (w/v) NaCl (aq) (1 L) and stirred (magnetic stirrer) at room temperature for 1 h. If any undissolved trypsin was present it was removed by filtration through Whatman No. 1 filter paper. The trypsin solution was then sterilised by filtration and dispensed into 10-20 mL aliquots and stored at -20℃. Ethylenediaminetetraacetic acid (EDTA) (3.72 g) was dissolved in to PBS (1 L) and sterilised by autoclaving. This PBS/EDTA (PE) solution was diluted 10-fold with PBS, and 90 mL of the diluted PE was mixed with 10mL of trypsin solution to produce the final trypsin solution. This solution was stored at 4℃ for a maximum of 3 weeks.

2.2.2. Cell Culture

2.2.2.1. Cell Seeding

One 100 µL cryovial containing approximately 5 x 10^5 rat testicular cancer Leydig cells, either R2C or LC-540, was removed from storage in liquid nitrogen and defrosted at room temperature (10-15 min). Culture medium (30 mL) was pipetted into a T-75 sterile culture flask using a sterile 10 mL glass pipette. The 100 µL cell suspension in the cryovial was added to the culture flask using a sterile micropipette with sterile tips. The flask was closed, removed from the sterile environment, and incubated at 37℃ in a 5% CO_2 atmosphere.

2.2.2.2. Passaging (splitting) Cell Cultures

Sterile Pasteur pipettes under vacuum suction were used to aspirate medium from the culture flasks, leaving the adherent cells behind. The flasks with adherent cells were rinsed with 2-4 mL PBS. Either 3 mL TrypLE express (A protease mix preprepared by Life technologies that specifically cleaves the C-terminal side of Lysine and Arginine) or 2 mL prepared trypsin solution (see 2.2.1.10.) was added to the flasks. The flasks were returned to the incubator for 5-15 mins until the cells were detached, this was observed using an inverted microscope. Sterile culture medium (10 mL) was added to inactivate the trypsin/TrypLE. The cell suspension was then transferred to 50 mL plastic centrifuge tubes and centrifuged at 4000 xg for 5 mins. The medium and trypsin/trypLE were aspirated (see above) from the centrifuge tubes and the pellets were resuspended in enough medium to seed the desired number of flasks (e.g. 3 flasks per tube corresponds to 30 mL medium per tube). Each flask had 20 mL of medium and 10 mL of cell suspension added using sterile pipettes. Flasks were incubated at 37℃ in an atmosphere containing 5% CO_2.

2.2.2.3. Cell Counting

The cell suspension for counting (20 μL) was mixed with trypan blue (80 μL, 4 mg/mL dissolved in PBS), this solution was mixed and 10 μL was pipetted to the top grid of a cytometer (0.1 mm deep, area = 0.04 mm^2) and a second 10 μL was pipetted to the bottom grid. A cover slip was placed over the cytometer and it was viewed at using an inverted microscope at 100x magnification. Each corner square on the cytometer's grids was counted and recorded (8 squares total) and the following formulae were used to determine the cell concentration:

$$T = C\ x\ \left(\frac{D}{N}\right)$$

$$T\ x\ 10^4 = Cells/mL$$

T = average number of cells in 1 x 10^{-4} mL; C = total cells counted; D = dilution factor; N = number of cytometer corner squares counted.

2.2.2.4. Growth Curves

Forty-five wells were seeded in three 15 well sets using three flasks. For each set the flask was treated with trypsin solution and centrifuged as stated previously (see section 2.2.2.2.). Each cell pellet was resuspended in 4 mL of medium (17.5% stripped FBS medium for R2C cells, 10% stripped FBS medium for LC-540 cells) and the cells were counted using a cytometer as stated previously (see section 2.2.2.3.). Based on the cell count, enough medium was added to give a cell concentration of 10^5 cells/mL (5 x 10^4 cells/well). Wells were seeded with 200 μL cell suspension. Medium (1.8 mL) was added to each well (repeated for the second and third set of 15 wells) resulting in a final starting cell concentration of 10^4 cells/mL. Every 24 hours, the culture medium from 3 wells was aspirated and 150 μL trypsin solution added; wells were incubated at 37 ℃ at 5% CO_2 for 5-10 mins, 350 μL of medium

was added to inactivate the trypsin, and wells were counted (as stated in section 2.2.2.3) for ten-fifteen days (depending on speed of growth) and cell counts were plotted against time to produce a growth curve.

2.2.3. Extraction of Steroids

2.2.3.1. Cell Lysate Extraction

Cells were lysed by centrifugation at 12,000 xg, the cell lysate was suspended in 500 µL of MilliQ water. Analytical grade diethylether (ether) (6 mL) was pipetted to a test tube with a glass stopper (to prevent evaporation). The cell lysate was added to the ether, the stopper was attached and the tube was inverted 10 times allowing the ether gas to escape by briefly loosening the stopper after each inversion. The mix was then given 2 min to separate into ether (top layer) and aqueous (bottom layer) phases. The ether layer was carefully removed with a Pasteur pipette and transferred into an open 15 mL test tube. The aqueous layer was then washed with another 6 mL ether and the inversion procedure was repeated two more times. The now separated ether layer was evaporated at 38℃ under nitrogen gas flow until all ether was evaporated. Samples were then resuspended in 1.5 mL Analytical grade methanol (MeOH) and filtered using a non-sterile syringe filter (0.45µm) into a 1.5 mL glass HPLC vial prior to analysis.

2.2.3.2. Cell Culture Medium Extraction

The culture medium (30 mL) was defrosted at room temp (30 min). ether (60 mL) was measured into a separating funnel with glass stopper. The medium was added to the ether in the funnel and the stopper replaced. The funnel was then inverted 10 times allowing ether gas to escape by briefly loosening the stopper after each inversion. The mixture was allowed to stand (2 min) to separate the ether (top layer) and aqueous (bottom layer) phases. The

aqueous layer was run off into a 100mL beaker. The ether layer was then run off into a 250 mL beaker. Another 60 mL of ether plus the aqueous layer (see 2.2.3.1.) was added to the separating funnel and the funnel inverted as described above. This was repeated twice. After the third separation the aqueous layer was discarded. Following each separation the ether phase was combined with the previous ether phases in the 250 mL beaker. The combined ether layers were evaporated in a 250 mL beaker standing in warm water until approximately 15 mL of ether remained. The ether was then swirled around the sides of the beaker to remove any residue that had formed during evaporation, and transferred to a test tube (if any aqueous layer contamination was seen in the test tube the ether layer was carefully removed using a Pasteur pipette as described in 2.2.3.1. and moved to another test tube) and was evaporated at 38℃ under a nitrogen gas flow. The residue was dissolved in 1.5 mL MeOH and filtered into a HPLC vial prior to analysis.

2.3. Analytical Techniques

2.3.1. High Performance Liquid Chromatography (HPLC)

Samples were analysed for steroids by reverse phase (C_{18}) HPLC using a step gradient elution profile and UV absorbance detection via photodiode array (210, 245, 280 nm). Each sample had 50 μL injected per analysis and each injection batch included a MeOH blank and a no injection elution solvents only blank.

2.3.1.1. HPLC System and Preparation

A Dionex brand HPLC system was used for all analyses. The HPLC needle was washed using MeOH and primed 5 times with MeOH. The HPLC system was then purged with a solvent mixture comprising 10% Acetonitrile, 90% MilliQ water containing 0.05% v/v trifluoroacetic acid (3 mins at 3 mL/min).

2.3.1.2. HPLC Column

A C_{18} Jupiter® reverse phase column was used to separate steroids

2.3.1.3. Mobile phase

Two elution buffers were used: Buffer A; MilliQ water containing 0.05% v/v trifluoroacetic acid, and buffer B; HPLC grade Acetonitrile. The following step gradients were used:

10% standard gradient:

90%A 10% B (0-2 min), 90%A 10%B-25%A 75%B (2-14 min), 25%A 75%B (14-24 min), 25%A 75%B-0%A 100%B (26-30 min), 0%A 100%B (30-32 min), 0%A 100%B-90%A 10%B (32-40 min), 90%A 10%B (40-42 min).

55% Acetonitrile gradient:

90%A 10% B (0-2 min), 90%A 10%B-50%A 50%B (2-18 min), 50%A 50%B (18-34 min), 50%A 50%B-0%A 100%B (34-35 min), 0%A 100%B (35-41 min), 0%A 100%B-90%A 10%B (41-46 min), 90%A 10%B (46-50 min).

2.3.2. Calibration Graphs

A 1 mg/mL standard of each analysed steroid was made up in MeOH and a 10-fold dilution series was prepared resulting in the following concentrations: 1 mg/mL, 0.1 mg/mL, 0.01 mg/mL, 1 μg/mL, 0.1 μg/mL, and 0.01 μg/mL. These standards were then filtered and injected onto the column along with a MeOH blank and eluted with either the 10% standard gradient or the 55% Acetonitrile gradient (see 2.3.1.3.) and the steroids detected by UV absorbance at 210 nm, 245 nm, and 280 nm. The peak areas at their 'ideal' wavelengths (i.e. the wavelength which gave the optimum peak area) was correlated against concentration to

give a calibration graph and the coefficient of determination (R^2) was used to determine the accuracy of the standard curve (must be 0.99 or higher). The point at which a peak was no longer observable was deemed the limit of analytical detection.

2.3.3. Limit of Determination

A 1 mg/mL standard of each steroid was diluted appropriately and added to culture medium extract to give final concentrations of 5 ug/mL, 1 ug/mL, 500 ng/mL, 400 ng/mL, 300 ng/mL, 200 ng/mL, or 100 ng/mL. These were injected onto the column along with a blank sample containing 10 μL MeOH. The lowest concentration at which the steroids could be detected was deemed the limit of determination (LoD).

2.3.4. Spiked Samples

To confirm the presence of a particular steroid, 100 μL of the sample being analysed was mixed with 100 μL of a standard concentration of steroid (e.g. 0.1 mg/mL cortisol producing a final concentration of 0.05 mg/mL cortisol in the sample). The peak area was measured and compared with the peak area of the steroid standard concentration. If the peak was larger and there were no peak shoulders the sample was identified as the standard steroid (i.e. the substances co-chromatographed).

2.4. Dibutyl Phthalate Exposure

Cell lines were passaged (see section 2.2.2.2.) into 12 T-75 flasks using phenol-red free medium and incubated until an approximate cell concentration of 5 x 10^5 cells/mL was reached (this took 5 days). Their media were replaced with medium containing either 300 μL of ethanol (control flasks), or a specific concentration of DBP suspended in ethanol (total volume of 300 μL) (the following final DBP concentrations were covered in two separate experiments: 2.44 mg/mL, 0.287 mg/mL, 0.10 μg/mL, 5 μg/mL, 1 μg/mL, 0.1 μg/mL, and

0.086 μg/mL). Each concentration as well as the control was done in triplicate. Prior to exposure, cell cultures were viewed microscopically and observations of their morphology and general density were noted. Similar observations were made immediately after DBP exposures and every 24 hours until the cells were harvested. Cells were exposed to DBP for a total of 72 hours and then the medium was collected and frozen at -20 ℃ until extracted as stated previously (see section 2.2.3.2.).

The cells were then treated with either TrypLE or prepared trypsin (3 mL) for 5-15 min to detach the cells. Culture medium (10 mL) was added to the flasks to inactivate the trypsin/TrypLE. The medium and cells were transferred to 50 mL plastic centrifuge tubes and the cells counted (see section 2.2.2.3.) and total cell number determined based on the cell suspension's total volume. The cells were lysed by centrifugation at 12 000 xg; the medium was removed and replaced with 500 μL of MilliQ water. Steroids were extracted from the cell lysate using the ether extraction procedure described previously (see section 2.2.3.1.). The resulting cell extracts were analysed using HPLC procedure described previously (see section 2.2.1.). Samples below the limit of detection were considered zero.

2.5. Statistical Analysis of DBP Exposed Samples

Data from each experiment was corrected to account for blanks by subtracting the average control peak area of each experiment from all other peak areas in that respective experiment. Experimental datasets were merged into one dataset and subjected to one way analysis of variance followed by Dunnett's multiple comparisons testing. Natural logs of data were used for all statistical analyses to account for increased noise at high DBP doses due to DBP's cytotoxicity. Differences were regarded as statistically significant at $P < 0.05$.

2.6. Exposure of LC-540 Cells to Cyclic AMP and Cyclic AMP Analogues

Cell lines were passaged (see section 2.2.2.2.) into 3 T-75 flasks using phenol-red free medium and incubated (37 ℃ 5% CO_2) until an approximate cell concentration of 5 x 10^5 cells/mL was reached (this took approximately 5 days). Two flasks were exposed to either cyclic AMP or N^6,2-O-Dibutyryladenosine3',5'-cyclic monophosphate (DB-cAMP) (suspended in culture medium) at 0.5 and 1 mM respectively. The third flask was used as a control and was not exposed. Flasks were incubated for 48 hours and then harvested and the culture medium was extracted with ether as described previously (see sections 2.4. and 2.2.3.2.).

2.7. Phenol Red-Free FBS Stripped Culture Medium vs Regular Culture Medium

To investigate the effect of phenol red and the hormone content of unstripped FBS R2C, triplicate cell cultures were grown in either regular medium or FBS stripped phenol red-free medium. Cells were counted and lysed and the resulting lysate had its steroids extracted and analysed by HPLC using the 10% standard gradient elution protocol (see sections 2.2.3.1., 2.3.1.).

Chapter 3 - Results

3.1. Steroid Analysis

3.1.1. HPLC Calibration Graphs

Calibration graphs were prepared by diluting stock solutions of progesterone, testosterone, and cortisol (see section 2.3.2) all had R > 0.99 (Fig. 3.1). These calibration graphs were used to calculate steroid concentrations in sample extracts. The analytical limits of detection were 10 ng/mL (testosterone), 50 ng/mL (progesterone), and 50 ng/mL (cortisol).

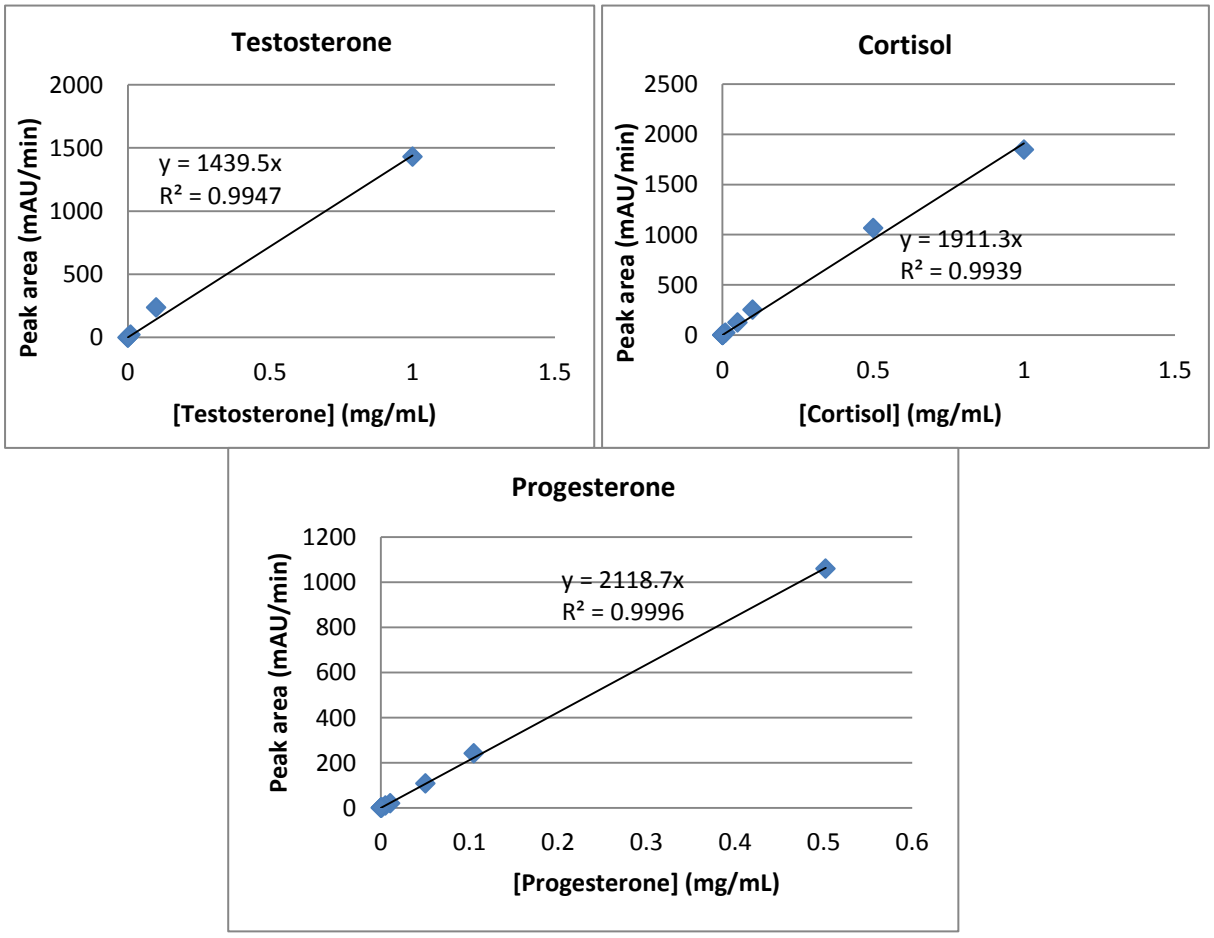

Figure 3.1: HPLC calibration graphs for testosterone, progesterone and cortisol. All steroid calibration graphs have $R^2 > 0.99$.

3.1.2. Limit of Determination

HPLC retention times (R_T) for steroids using the 55% acetonitrile elution gradient (2.3.1.3.) were used to determine if a quantifiable change had occurred in spiked samples: Progesterone (R_T = 22.8-22.9 min), testosterone (R_T = 18.8 – 18.9 min) and cortisol (R_T = 14.0 – 14.1 min) were analysed. All steroids had a LoD of 100 ng/mL (Table 3.1) which was the lowest spiked steroid concentration that gave a quantifiable change. This means that any steroid levels detected by HPLC below this limit are not valid and will not be considered significant.

[Steroid] (ng/mL)	**Peak area (mAU/min)**		
	Cortisol	*Testosterone*	*Progesterone*
0	5.43	0.2	0.03
100	5.59	0.5	0.28
200	5.66	0.62	*
300	5.85	1.19	*
400	5.99	1.14	*
500	6.01	1.52	1.83
1000	*	*	4.34
5000	*	*	16.08

*Table 3.1: LoD data for steroid-spiked culture media. All steroids were detectable at 100 ng/mL (5 ng injected). Progesterone was the first steroid tested and was analysed much more broadly to determine if steroids could be detected at ng concentrations or only µg concentrations. Testosterone and cortisol were analysed at ng concentrations. All steroids were detectable at 100 ng/mL. * = A concentration that was not tested (progesterone was the first steroid analysed so a broader range of concentrations were used than the later tested cortisol and testosterone).*

3.2. Cell Culture Characterisation

3.2.1. Growth Curves

Growth curves of both the R2C and LC-540 cell lines were collected using the same methodology (see section 2.2.2.4). Both R2C and LC-540 growth curves have clear lag, log, and death phases (Fig. 3.2). The LC-540 growth curve has a much longer lag time (10 days) than the R2C growth curve (5 days) (Fig. 3.2). The LC-540 growth curve has a peak cell number of approximately 1.5 x 10^5 cells/well whilst the R2C growth curve has a peak cell number of approximately 1 x 10^6.

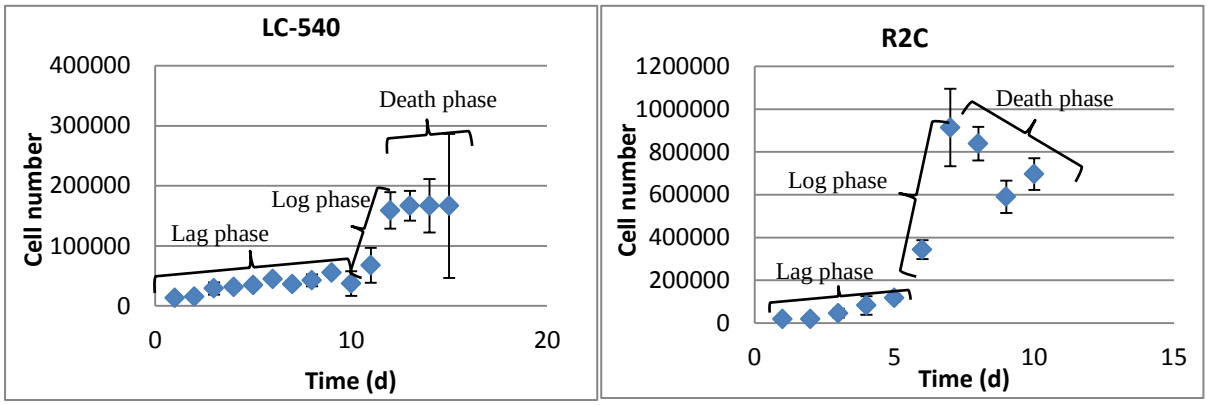

Figure 3.2: Growth curves for LC-540 and R2C cell lines (mean ± SD). Lag, log and death phases in cell growth are labelled. LC-540 cells have a much longer lag time than R2C cells.

3.2.2. Cell Description

R2C cells can be either oval or spherical when adhered to the culture vessel surface (Fig. 3.3A). As R2C cell clusters become denser new cells grow on top of older cells until conglomerates are formed which are visible to the naked eye. LC-540 cells are quite different, they form a very thin widely spread monolayer when adhering to surfaces (Fig. 3.3B). This monolayer is not visible to the naked eye. When LC-540 cells are treated with trypsin the monolayer peels off in a single sheet before the cells separate from one another, making the monolayer visible. When they are not adhered both LC-540 and R2C cells are spherical (Fig. 3.3C).

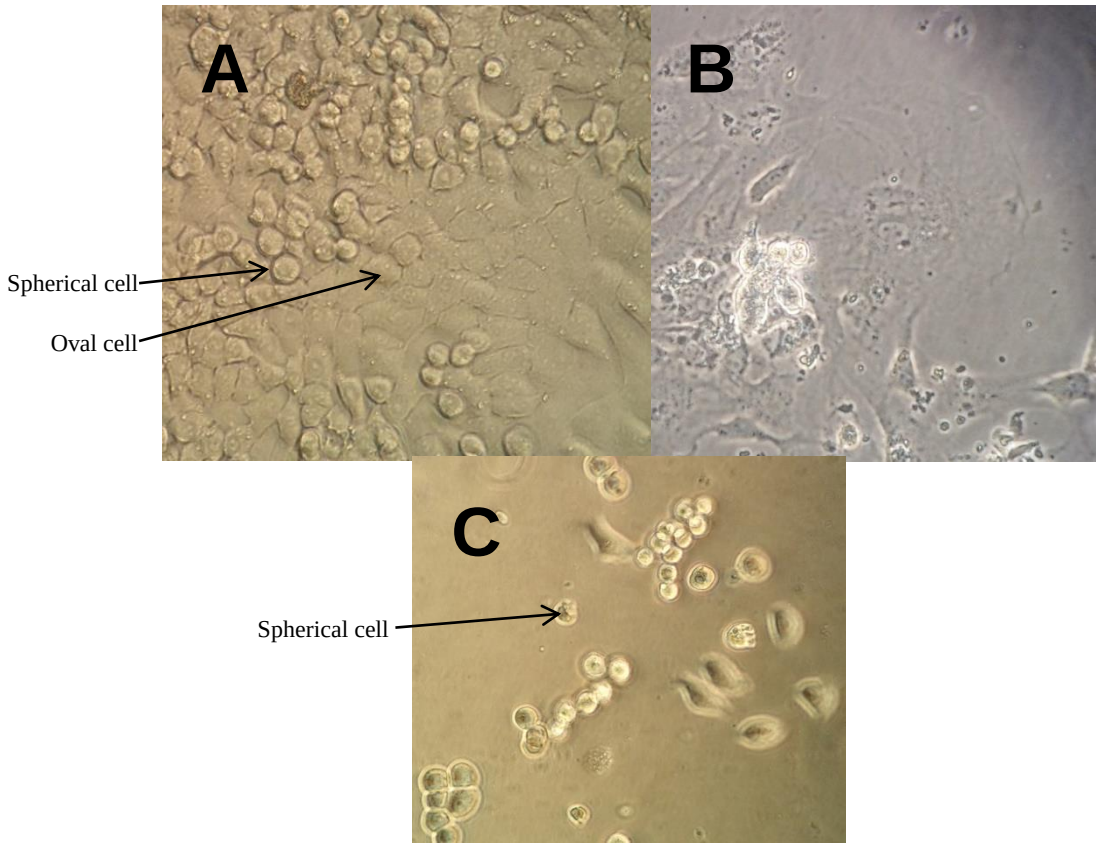

Figure 3.3: Images of cells in culture in T-75 culture flasks at 100x magnification. ***A****: R2C cells at confluence adhered to a culture flask. Both spherical and oval cells can be seen adhered to the flask.* ***B****: LC-540 cells at confluence adhered to a culture flask. Cells are more spread out when adhered to the flask when compared with the R2C cells.* ***C****: R2C cells following the trypsin detachment process. Cells are spherical in shape. LC-540 cells also look like this when exposed to trypsin.*

3.3. Phenol Red-Free FBS Stripped Culture Medium VS Regular Culture Medium

Phenol red is a commonly used indicator of cell viability which reacts to CO_2 production indicating when cell overpopulation or contamination has occurred. However, phenol red is a known xenoestrogen and thus could cause changes in steroid biosynthesis. FBS is known to contain trace levels of steroids which can be removed using serum stripping (see section 2.2.1.3.). To determine whether the removal of phenol red and these trace steroids caused a significant change in steroidogenesis the steroids in R2C cells grown in both standard culture media and phenol red-free FBS stripped culture media were analysed for significant

differences which were found. Progesterone levels were higher and cholesterol levels lower in extracts from cells grown in standard culture media compared with those grown in phenol red-free FBS stripped culture media (Fig. 3.4, 3.5). This suggests that either phenol red or FBS steroids are inducing the stimulation of steroidogenesis because progesterone is the R2C cell line's main steroid product and cholesterol is a universal steroidogenesis precursor. Because of this, all future samples will be grown in FBS stripped phenol red-free media to remove this background stimulation.

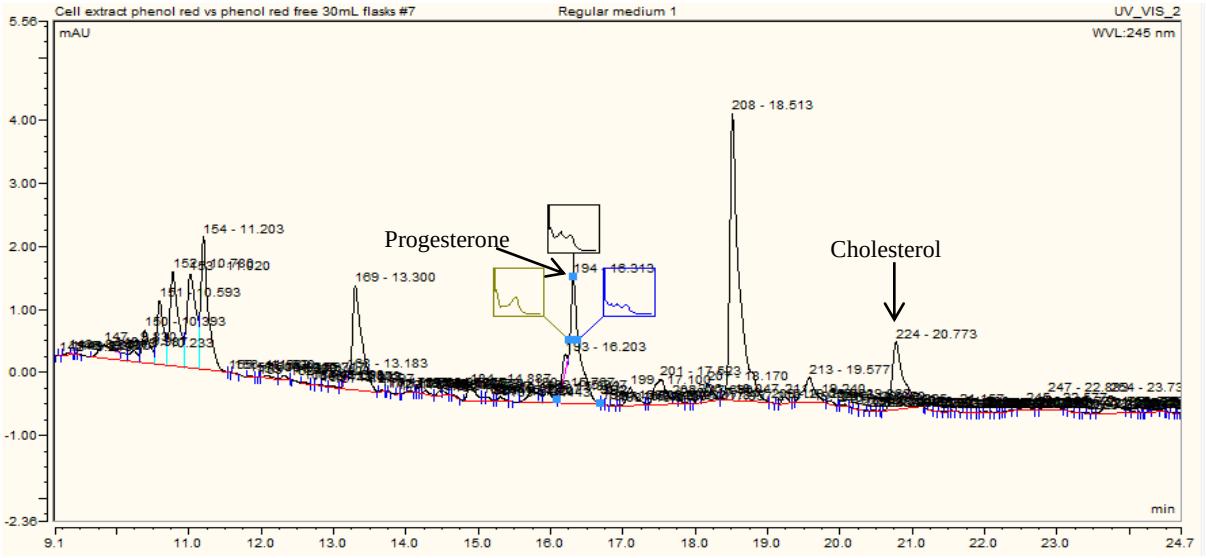

Figure 3.4: HPLC trace of R2C cell lysate extract (cells were grown in standard 17.5% MEM) measuring the absorbance at 245 nm. Peaks at 16.313 and 20.773 mins correlate to progesterone and cholesterol respectively.

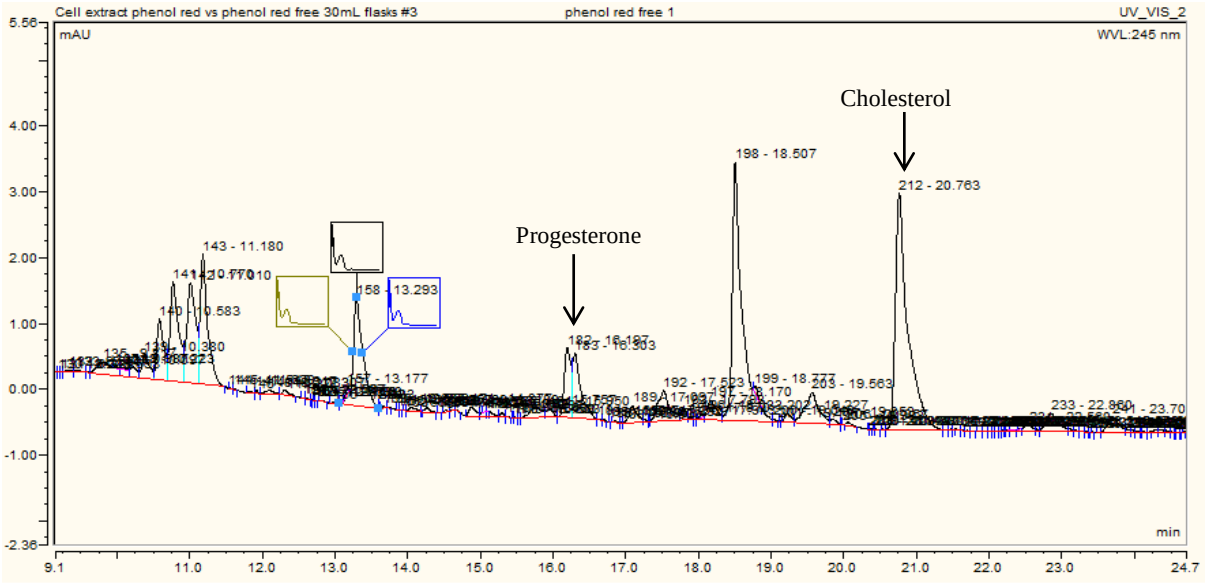

Figure 3.5: HPLC trace of R2C cell lysate extract (cells were grown in Serum stripped 17.5% phenol red-free MEM) measuring the absorbance at 245 nm. Peaks at 16.313 and 20.773 mins correlate to progesterone and cholesterol respectively.

3.4. Determining Analytical Background Noise

3.4.1. Stripped Media Analyses

Stripped culture medium was analysed using HPLC chromatography standard 10% elution gradient (see section 2.3.1.) and the HPLC trace was analysed for any peaks correlating with known steroid retention times (Fig. 3.6). No peaks correlating with testosterone, progesterone, and cholesterol were seen but a peak correlating with cortisol was seen. However, the UV spectrum of the peak does not match with cortisol's UV spectra (Fig. 3.7).

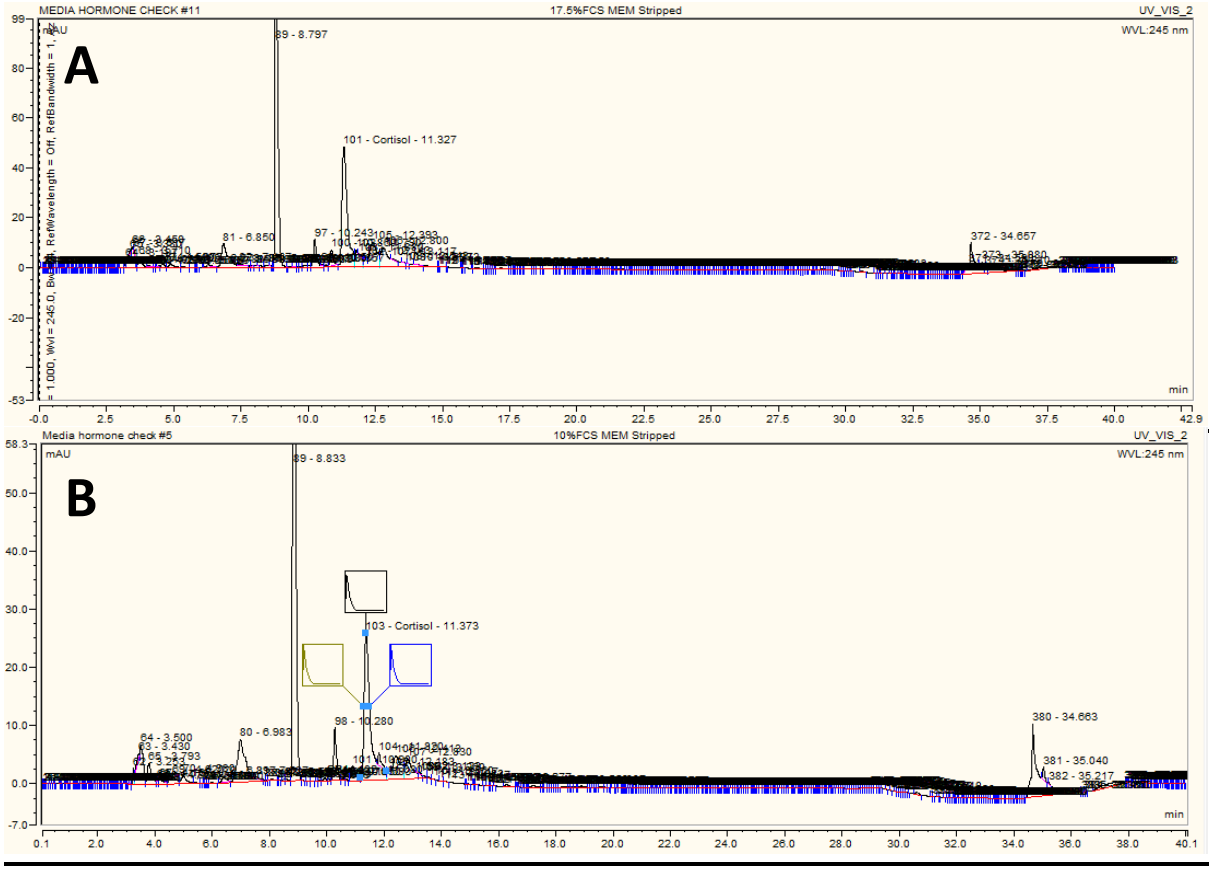

Figure 3.6: HPLC UV absorbance trace of stripped media measured at 245 nm. ***A****: 17.5% stripped FBS phenol red-free MEM (used with R2C cells).* ***B****: 10% stripped FBS phenol red-free MEM (used with LC-540 cells). Peaks correlating with cortisol can be seen in both samples of medium.*

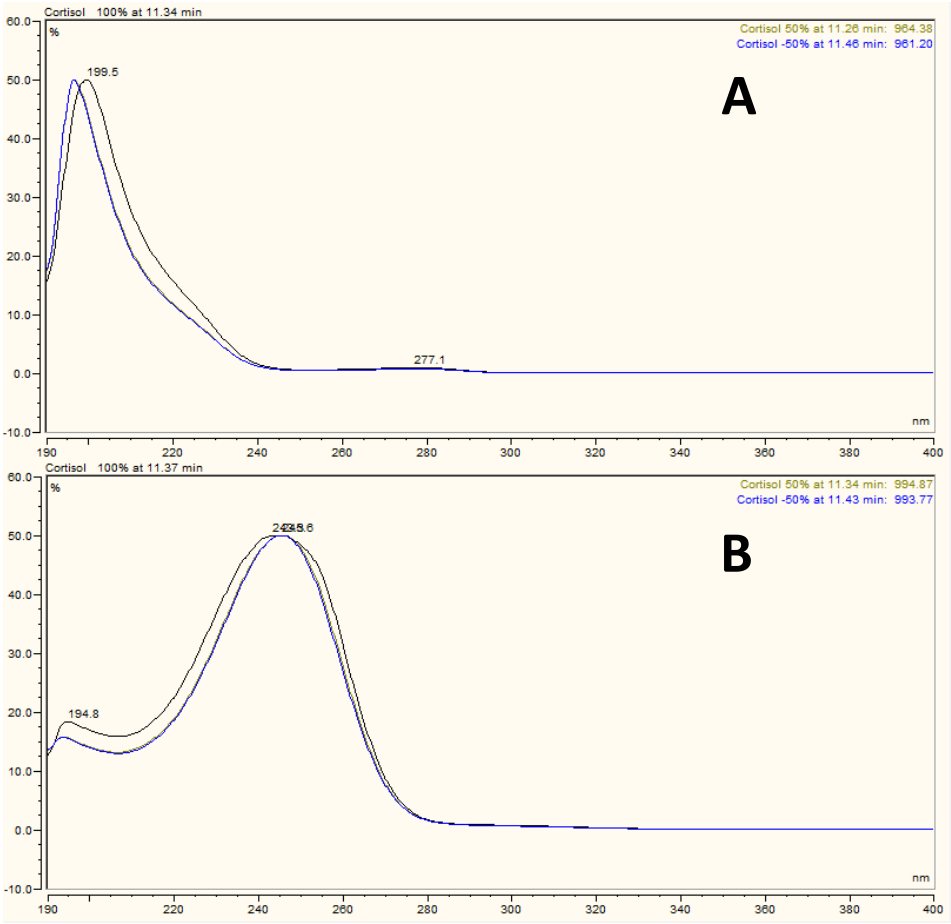

Figure 3.7: UV spectra of cortisol compared with the peak found in medium. ***(A)*** *The UV spectra of the peak in stripped FBS media with the same retention time as cortisol, peaking in absorbance at approximately 200 nm. (****B****)The UV spectra of a standard 1 mg/mL cortisol sample. The primary absorbance peak for the cortisol standard is at approximately 245 nm.*

3.4.2. Ether Extraction Analysis

Media was extracted using ether (see section 2.2.3.2.) and 180 mLs of pure ether was evaporated in triplicate as stated in section 2.2.3.2., analysed using HPLC (see section 2.3.1.) and compared (Fig. 3.8). The peaks observed are identical between the two samples showing that all background noise in analysis is a result of compounds found in the ether residue.

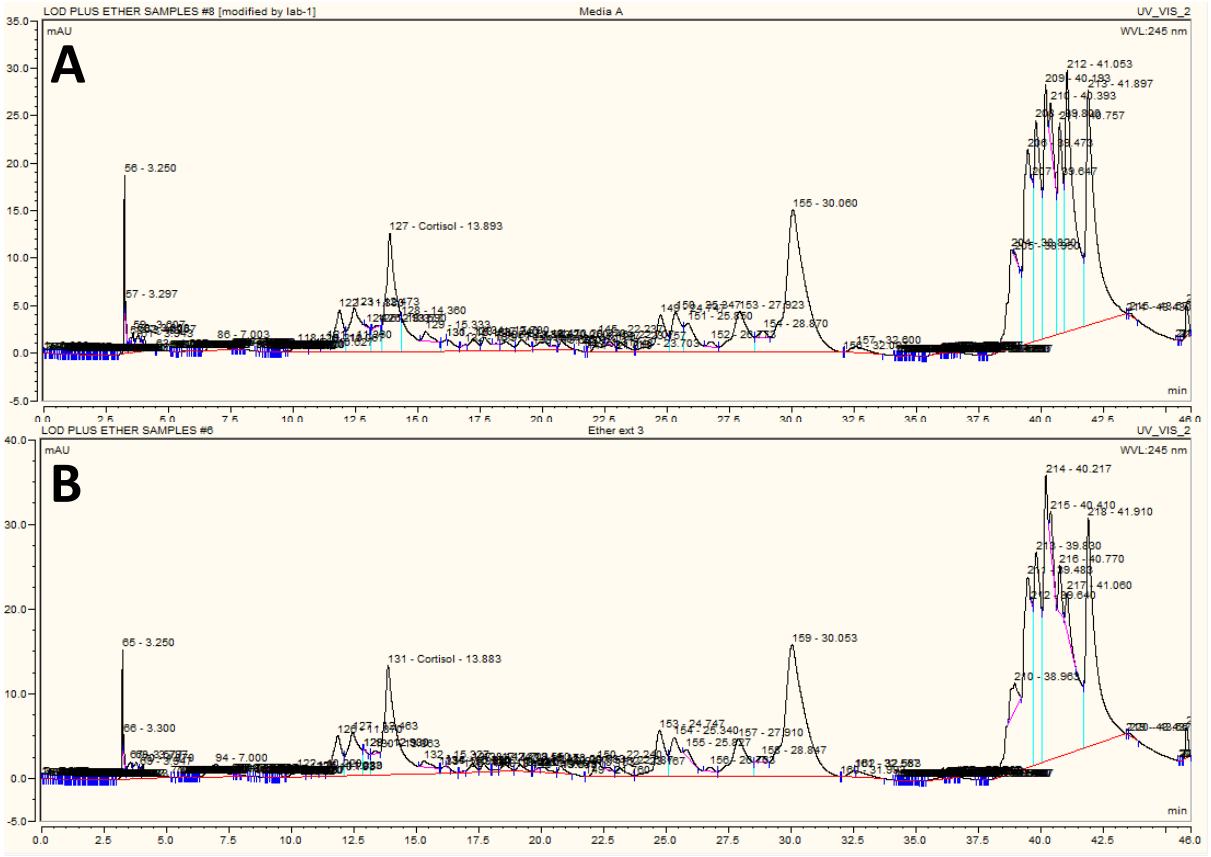

Figure 3.8: HPLC trace measuring of both extracted media and 'ether' residues absorbance at 245 nm. Both traces are identical. ***A****: UV trace of fresh media extract.* ***B****: UV trace of 'ether'.*

Cortisol background noise levels are constant between samples as the three ether samples have identical peak areas for the peak associated with cortisol (Table 3.2). On the other hand, there is variation in background noise between bottles of ether. However, because a maximum of one ether bottle was used in each experiment, any changes in steroid peaks within that experiment are significant. To account for this non-cortisol background noise, the average cortisol peak area of the control samples of each exposure experiment were subtracted from all other cortisol peak area values (control samples had the lowest cortisol peak area in all exposure experiments).

Sample	**Peak area at R_T 13.8min (mAU/min)**
Ether extract 1	6.08
Ether extract 2	6.08
Ether extract 3	6.07

Table 3.2: Peak areas of 'ether' extracts at cortisol's retention time (R_T: 13.8). All samples had identical peak areas.

3.5. HPLC Method Development

Cell lysate was harvested and extracted from unexposed cells to confirm the presence of steroids (see section 2.2.3.1.) using the standard 10% HPLC elution gradient (see section 2.3.1.3.). Peaks co-chromatographing with progesterone (R_T 16.3) and cholesterol (R_T 20.8) were observed in samples (Fig. 3.9) (these peaks had their identities confirmed by comparing their UV spectra with known steroid spectra).

However, due to peak overlap, particularly with progesterone (Fig. 3.9), the elution protocol was altered to smooth out the gradient. This resulted in the 55% acetonitrile protocol with much more spread out peaks, thus increasing the accuracy of peak identification and quantification (Fig. 3.10).

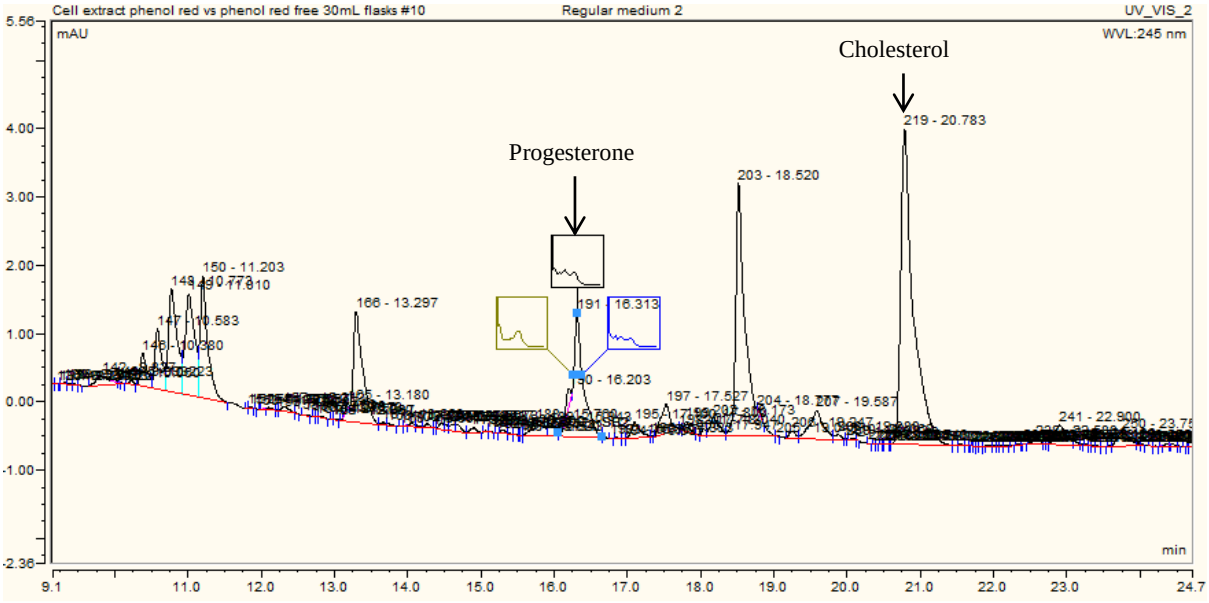

Figure 3.9: HPLC trace measuring UV absorbance at 245 nm of R2C cell lysate extract (cells were grown in standard 17.5% MEM). Peaks at 16.313 and 20.783 mins correlate to progesterone and cholesterol respectively. A small interfering peak can be seen next to the progesterone peak.

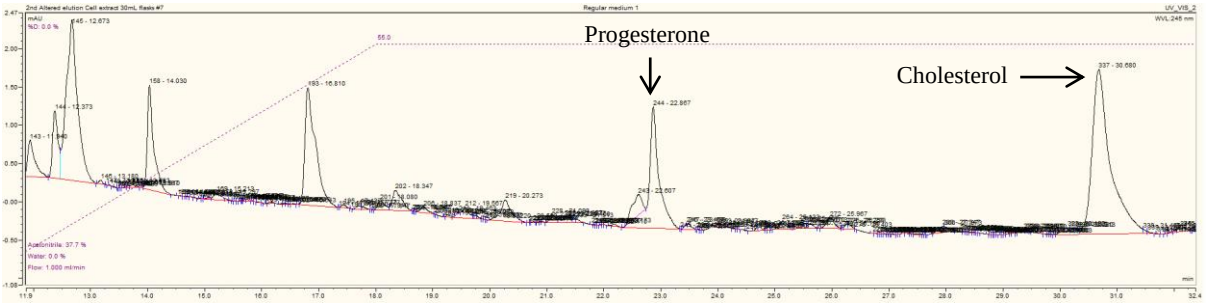

Figure 3.10: HPLC trace measuring UV absorbance at 245 nm of R2C cell lysate extract (cells were grown in standard 17.5% MEM) using the 55% acetonitrile elution protocol. Peaks at 22.867 and 30.680 mins correlate to progesterone and cholesterol respectively. A small peak can be seen next to the progesterone peak.

3.6. Cell Counting Method Development

The original TrypLE express mix proved ineffective at remove all cells attached to the flask and did not separate individual cells well resulting in cell clumping which causes inaccuracy in cell counts (Fig. 3.11A). To remedy this, trypsin was dissolved in solution (see section 2.2.1.10.). This trypsin solution resulted in the removal of all cells from the plastic surface

and produced much better separation between cells resulting in a much more accurate count (Fig. 3.11B). Dissolved trypsin powder was then used for all future experiments.

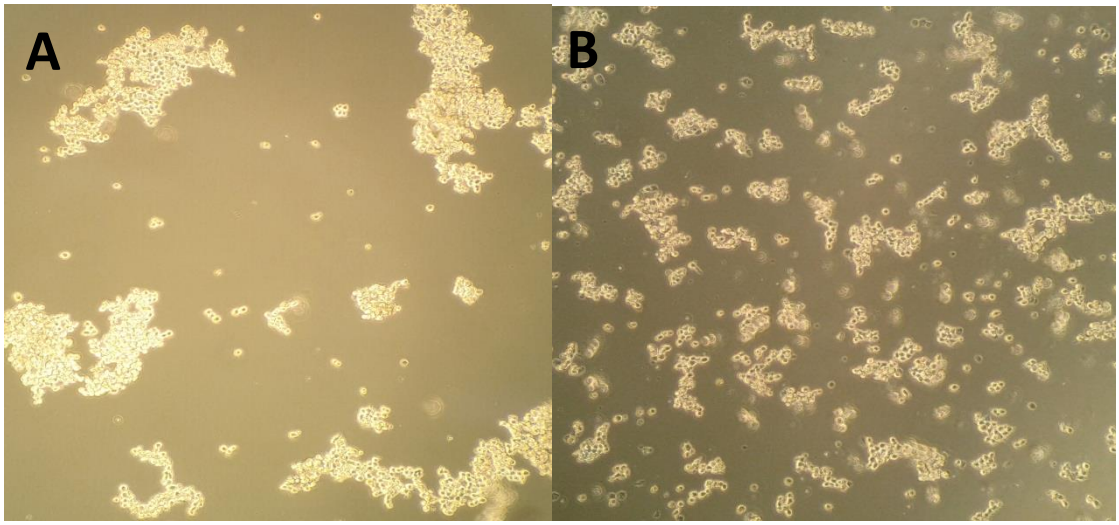

Figure 3.11: Cells detached from the surface of their culture flask using either: ***A****: trypLE express, or* ***B:*** *trypsin solution. Cells are much more clustered when using trypLE express.*

3.7. Cell Steroid Production

3.7.1. R2C Extractions

Unexposed R2C cells were grown for 5 days, had their media replaced, and were harvested three days later (see section 2.4.). Both the cell lysate and culture medium were extracted (see sections 2.2.3.1., 2.2.3.2.), analysed for steroid content (see section 2.3.1.) and compared (Fig. 3.12).

In both the cell lysate and medium extracts progesterone peaks can be seen (Fig. 3.12). The much larger peaks in the culture medium will be easier to identify and any changes will be a greater distance from the LoD making the culture medium extract a much more useful tool in analysing steroid content. A cortisol peak is also observed but this could be due to the background noise produced by the ether (see section 3.4.2.) so it cannot be conclusively

identified as cortisol at this time. However, because the background noise is constant (see section 3.4.2) any increases in this peak would be indicative of cortisol production.

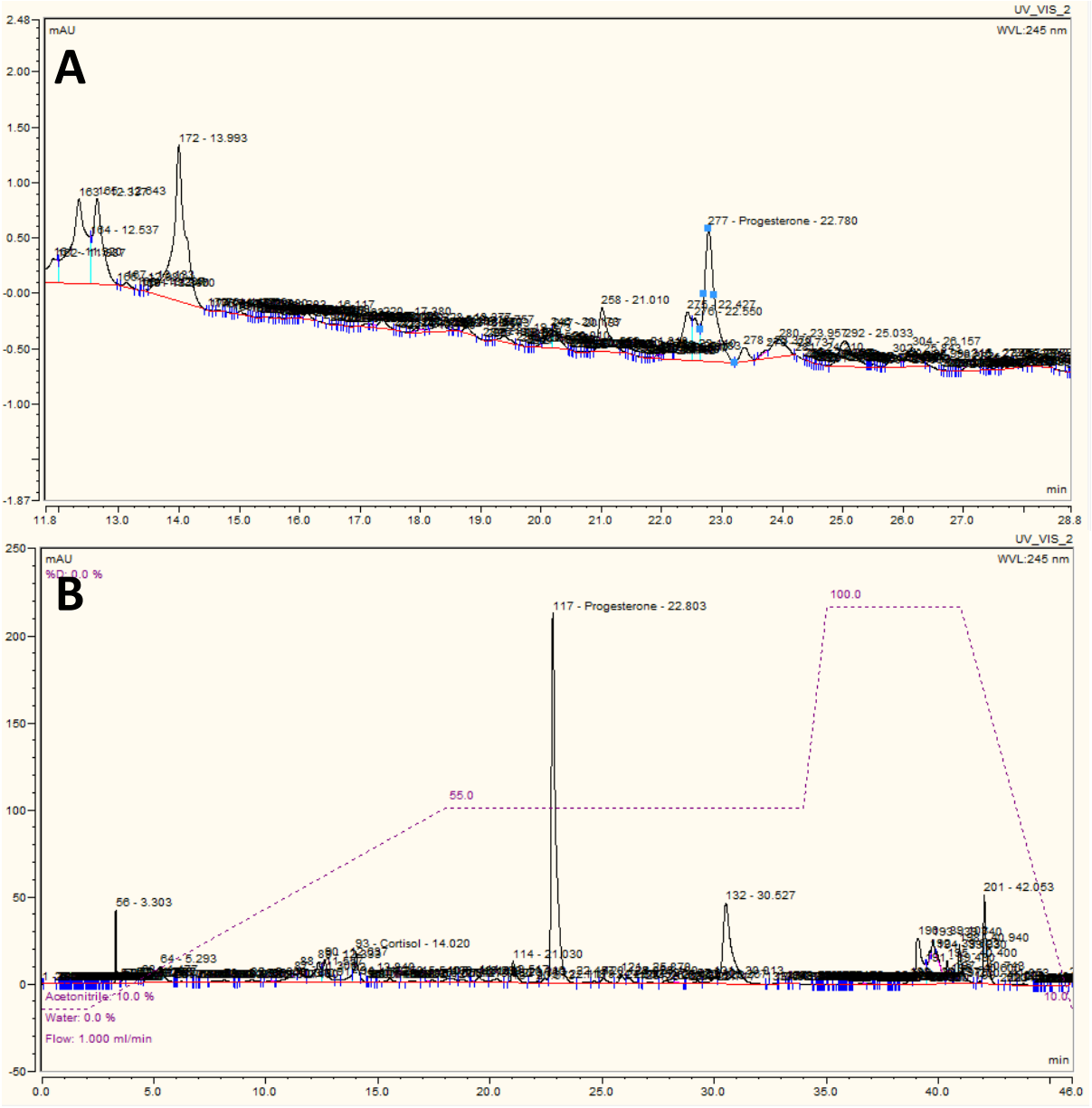

*Figure 3.12: HPLC trace showing UV absorbance at 245 nm from: **A**: R2C cell lysate extract, or **B:** R2C culture medium extract. Peaks with matching retention times to cortisol and progesterone are in both samples. Progesterone was confirmed present by comparing the UV spectra of the peak at 22.8 with the UV spectra of a 1 mg/mL standard (both peak in absorbance at 245 nm). Steroid peaks are much larger in extracted culture medium making the analysis of cells culture medium a much more effective way to quantify relative steroid production.*

3.7.2. LC-540 Extractions

Unexposed LC-540 cells were grown for 5 days, had their media replaced, and were harvested three days later (see section 2.4.). Both the cell lysate and culture medium were extracted (see sections 2.2.3.1., 2.2.3.2.), analysed for steroid content (see section 2.3.1.) and compared (Fig. 3.12).

In the culture medium extract both testosterone and progesterone peaks can be seen as well as a potential cortisol peak (Fig. 3.13). However, as with the R2C extracts the potential cortisol peak could be due to background noise produced by the use of ether so this cannot be conclusively identified as a cortisol peak. The LC-540 cells produce testosterone unlike R2C cells but they do not produce nearly as much progesterone as R2C cells. No significant steroid peaks can be identified in the cell lysate extract but testosterone and progesterone can be positively identified in culture medium extract (Fig 3.13). Because of this, culture medium extract will be used to identify and quantify steroids produced by the cells in future experiments.

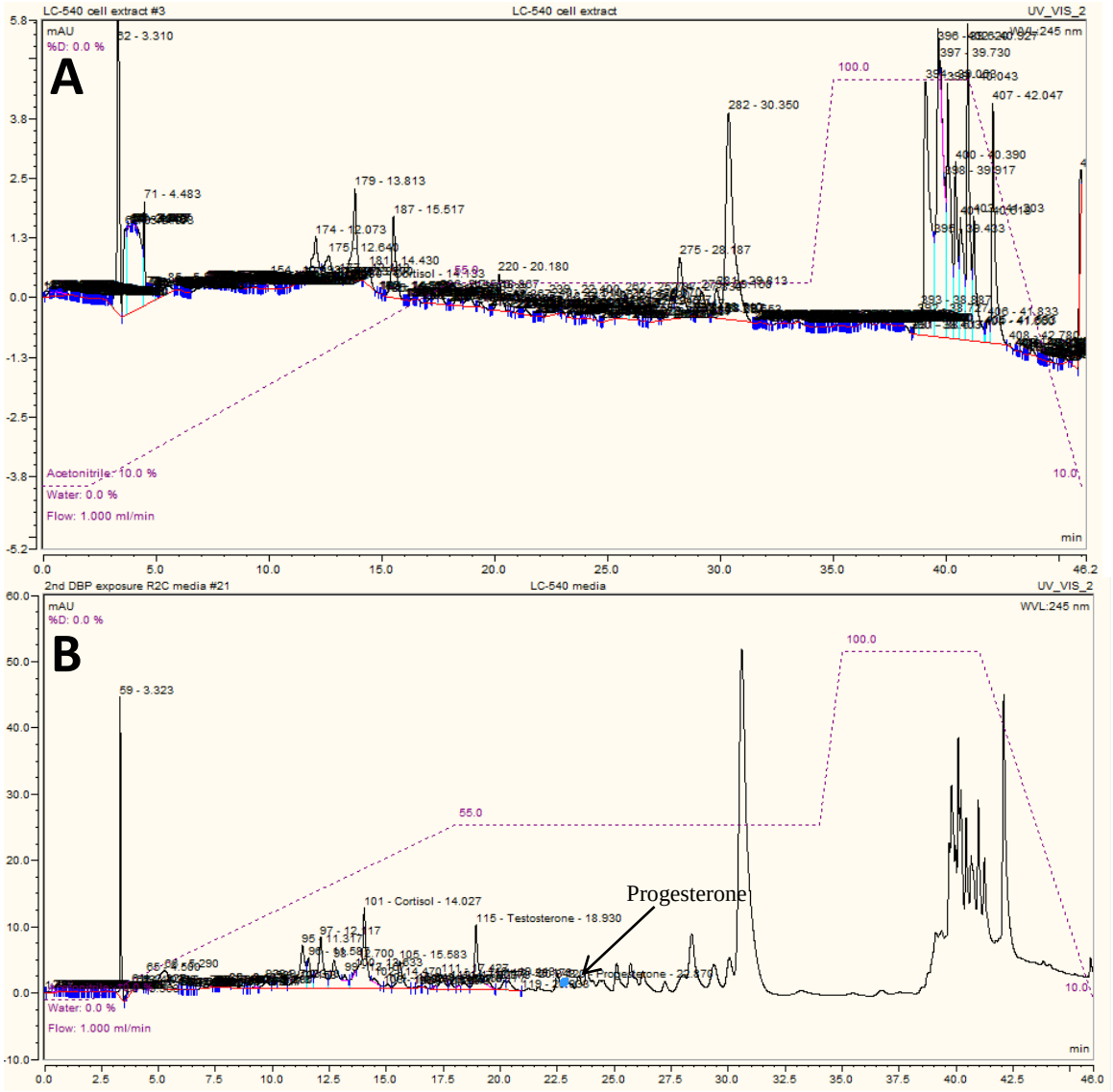

*Figure 3.13: HPLC trace measuring UV absorbance at 245 nm from: **A**: LC-540 cell lysate extract, or **B:** LC-540 culture medium extract. Peaks with matching retention times to cortisol and testosterone are seen in the extracted culture medium. Testosterone and progesterone were confirmed present by comparing the UV spectra of the peaks at 18.9 and 22.8 with the UV spectra of 1 mg/mL standards (all of which peak in absorbance at 245 nm). Like the R2C extract steroid peaks were much larger in the medium extract making media analysis a much more effective way to quantify relative steroid concentrations.*

3.8. Determining DBP Exposure levels

R2C cells were exposed to doses of 2.44, 0.287 mg/mL, and 0.086 µg/mL DBP (see section 2.4.) (high concentrations are estimations of DBP present in the blood of New Zealand soldiers based on Carran and Shaw's exposure study (assuming 70kg and 3.3L serum

volume) (Carran & Shaw 2012), low dose based on average adult dietary exposure in Denmark (assuming 70kg and 3.3L serum volume) (EFSA 2005)).

R2C cells exposed to levels of 0.287 and 2.44 mg/mL DBP have a large decrease in cell numbers. These high doses resulted in the formation of insoluble globules (Fig. 3.14), cortisol culture media levels that were too variable to be considered significantly different from the control (Fig. 3.15), and progesterone culture media levels that were significantly decreased (Fig. 3.16). However, this progesterone decrease could be due to the limitations of the cell count at low cell concentrations (1×10^4 is the lowest possible cell count, but cells may be at an even lower concentration). Samples exposed to 0.086 μg/mL doses of DBP showed no observable effect on cell number or cell morphology (Table 3.3, Fig. 3.14) as well as no significant change in cortisol or progesterone culture media levels (Figs. 3.15, 3.16). Testosterone was not detected (below the LoD) in any of the culture media analysed. In all future experiments samples were exposed to DBP levels below the saturation concentration of DBP in water (13 μg/mL) to avoid the formation of insoluble globules and above the lowest exposure tested (0.086 μg/mL) as it had no observable effect on cortisol or progesterone production.

DBP Exposure	**Rep 1 (cell/mL)**	**Rep 2 (cell/mL)**	**Rep 3 (cell/mL)**
0 mg/mL	2×10^5	1.9×10^5	2×10^5
8.6×10^{-5} mg/mL	2.6×10^5	1.4×10^5	2.4×10^5
0.297 mg/mL	9.4×10^4	3.7×10^4	5×10^4
2.44 mg/mL	1.8×10^4	1×10^4	1×10^4

Table 3.3: Cell counts of all samples 72 hours after exposure to DBP. Cell count is lower in high exposure samples.

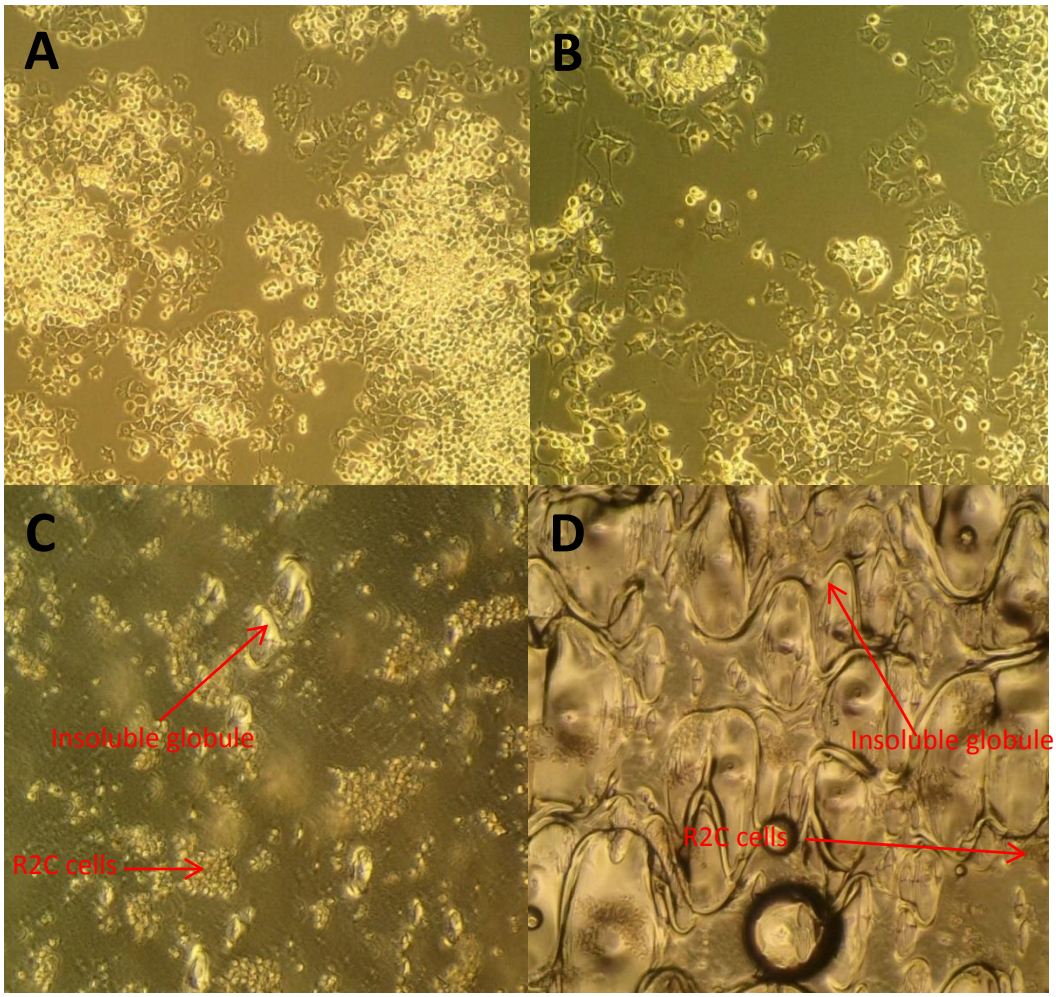

*Figure 3.14: Photographs of R2C cells exposed to various DBP concentrations after 24 hours. **A**: Control cells **B:** Cells exposed to 0.086 µg/mL DBP. These are very similar to control samples. **C:** Cells exposed to 0.3 mg/mL DBP. Small insoluble globules can be seen adhering to the surface of the flask. **D:** Cells exposed to 2.4 mg/mL DBP. Insoluble globules have almost completely covered the surface of the flask resulting in the majority of the R2C cells removal.*

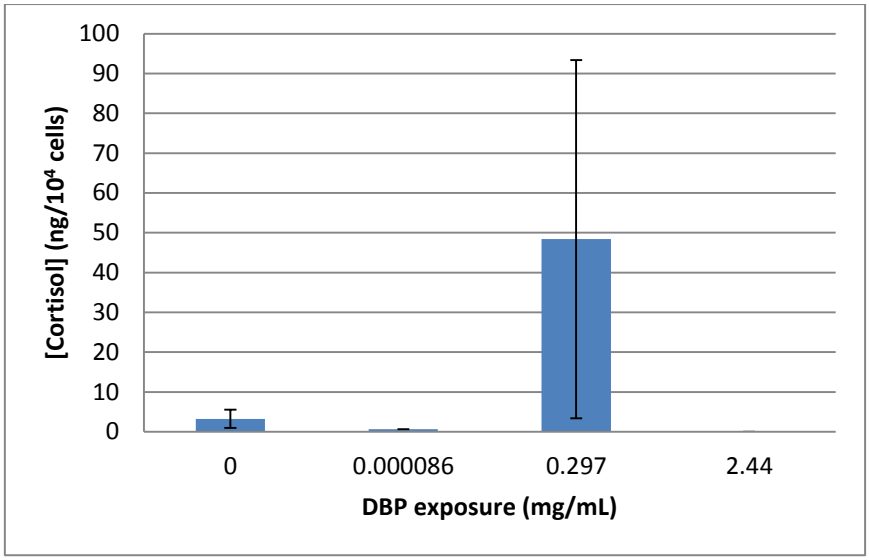

Figure 3.15: Cortisol levels in culture media from R2C cells exposed to DBP for 72 h showing DBP-induced biosynthesis of cortisol (error bars = standard deviation).

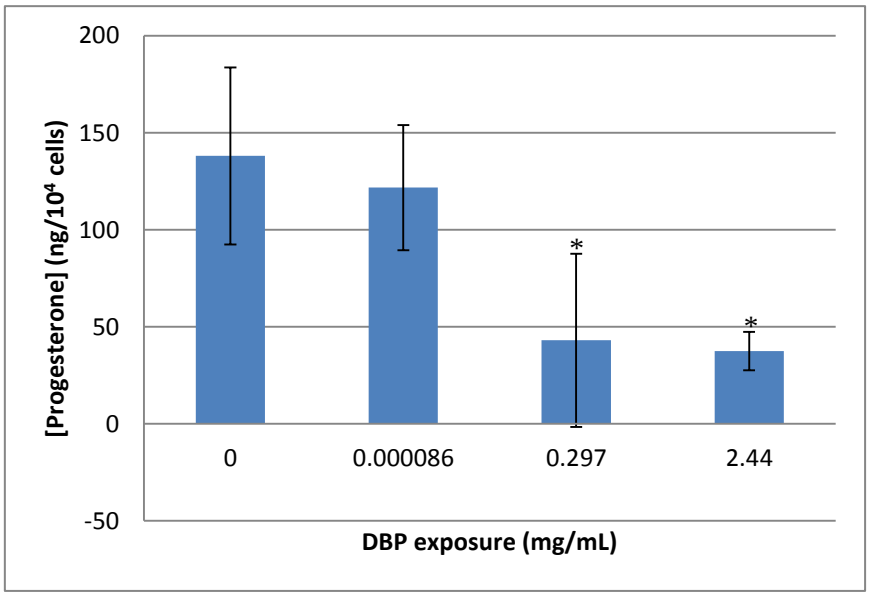

Figure 3.16: Progesterone levels in culture media from R2C cells exposed to DBP for 72 h showing a decrease in progesterone at cytotoxic concentrations(error bars = standard deviation) (= P<0.05 with respect to control).*

3.9. Exposure of R2C Cells to DBP

Following the previous exposure of R2C cells to a broad range of DBP doses (see section 3.8) a second exposure experiment was run using the same methodology (see section 2.4) exposing the R2C cells to a less broad range DBP doses (0.1, 1, 5, and 10 μg/mL). Cortisol

culture media levels in all DBP exposed R2C cells were significantly ($P < 0.05$) greater than control culture media levels (Fig. 3.17). Progesterone culture media levels were unaffected by DBP exposure (Fig. 3.18). Testosterone was not detected (below the LoD) in any of the culture media analysed.

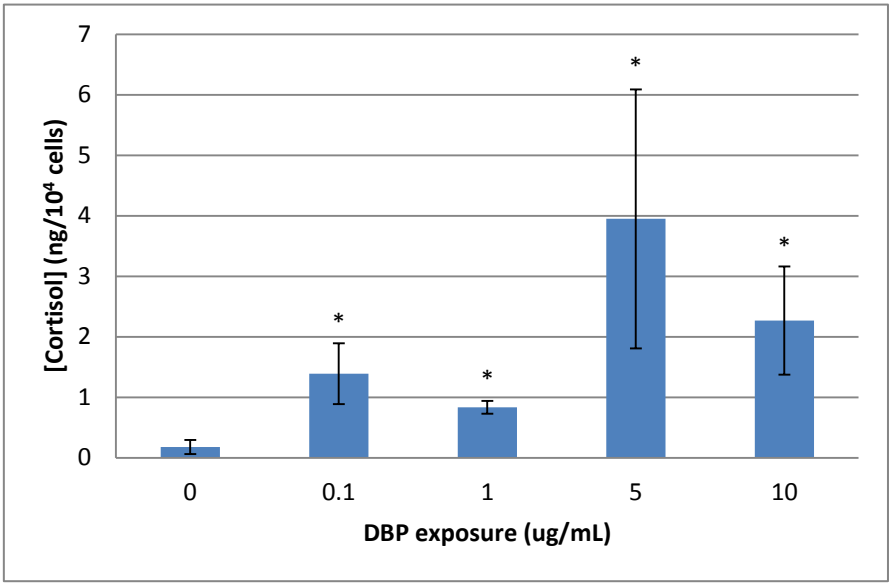

Figure 3.17: Cortisol levels in culture media from R2C cells exposed to DBP for 72 h showing DBP-induced biosynthesis of cortisol (error bars = standard deviation) (= P<0.05 with respect*

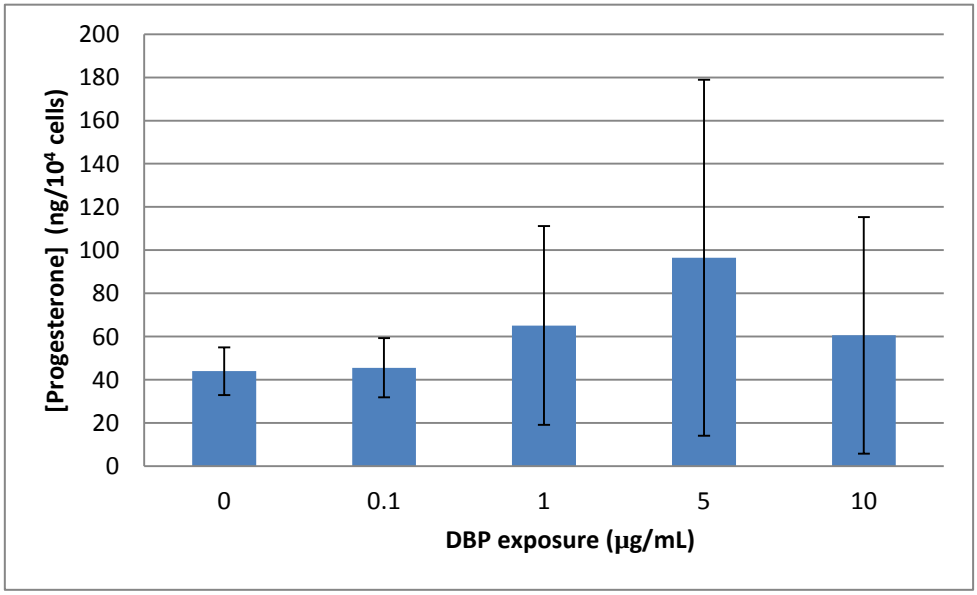

Figure 3.18: Progesterone concentrations in culture media from DBP-exposed R2C cells showing that DBP exposure did not affect the biosynthesis of progesterone (error bars = standard deviation).

3.10. Effects of Cyclic AMP in LC-540 Steroid Biosynthesis

Because the R2C cell line does not produce testosterone it is not the ideal cell line for analysis. However, the LC-540 cell line does produce detectable levels of testosterone. To further investigate this cell lines use as an accurate Leydig cell model cAMP's ability to regulate testosterone biosynthesis was investigated in a brief one-off experiment. In normal Leydig cells increased levels of internal cAMP upregulates steroidogenic enzymes resulting in increased testosterone biosynthesis. The cAMP analogue DB-cAMP was also investigated because it is known to permeate cell membranes efficiently. Testosterone peak areas do not significantly change in LC-540 cells exposed to cyclic AMP (Table 3.4). LC-540 cells exposed to 1mM DB-cAMP seem to more than double in their production of testosterone (Table 3.5). However, as this is a one off experiment this cannot be confirmed.

Sample	**Peak area at R_T 18.8min (mAU/min)**
LC-540 0mM cyclic AMP	2.0
LC-540 0.5mM cyclic AMP	2.3
LC-540 1mM cyclic AMP	2.1

Table 3.4: Peak areas of cyclic AMP exposed LC-540 culture medium extracts at testosterone's retention time (R_T: 18.8).

Sample	**Peak area at R_T 18.8min (mAU/min)**
LC-540 0mM DB-cAMP	1.8
LC-540 0.5mM DB-cAMP	1.7
LC-540 1mM DB-cAMP	4.4

Table 3.5: Peak areas of DB-cAMP exposed LC-540 culture medium extracts at testosterone's retention time (R_T: 18.8).

Chapter 4 - Discussion

4.1. A Brief Summary of Endocrine Disruption and Reproductive Development

4.1.1. Testosterone's Role in Male Development

Both testosterone and DHT are the key androgens involved in the expression of the male phenotype. During fetal development these hormones promote the formation of the Wolffian duct, male external genitalia and stimulate testes descent (Jost 1953; Yeh et al. 2002; Brown 1995; Russell & Wilson 1994; Hutson et al. 1997). DHT is synthesised from testosterone by 5α-reductase in specific tissues (Deslypere & Young 1992; Russell & Wilson 1994; Mahendroo & Russell 1999). The majority of the testosterone metabolised by 5α-reductase (as well as most of the testosterone used to express male characteristics throughout the body) is synthesised by testicular Leydig cells. These Leydig cells are the primary source of testosterone in the male (Christensen & Mason 1965; Wattenberg 1958) though testosterone is also secreted in small amounts by the adrenal gland (Tanabe et al. 1979). Therefore, any change in Leydig cell testosterone secretion (particularly during early development) will affect an individual's male characteristics. The degree of this effect will be directly related to the change in testosterone exposure. For example, detrimental effects can be seen in those with androgen insensitivity syndrome; this can be caused by a number of deleterious mutations on the AR gene (these can be complete removal of the AR gene via an early stop codon or point mutations interfering with the androgen binding domain or the DNA binding domain) (Brown 1995). Those who suffer from this condition can have a variety of developmental disorders ranging from infertility to muscular atrophy during adulthood in mild cases to expressing the female phenotype in severe cases (Brown 1995).

4.1.2. Endocrine-Disrupting Chemicals

EDCs interfere with the endocrine system which includes the male hormone testosterone and the female hormone 17β-estradiol. These compounds are very common and can disrupt steroid hormone activity in a variety of ways. Some mimic their associated hormones. For example, 17β-estradiol mimics bind to the ERs increasing estrogenic activity. Activated ERs stimulate the expression of the female phenotype stimulating the division of cells in female tissue and supress the androgen production of Leydig cells (Akingbemi 2003; Kalla et al. 1980). Other EDCs can cause the adverse effects by targeting hormone activity. For example, antiandrogens can target the AR and occupy the binding site so that testosterone and DHT cannot bind reducing androgen activity and male phenotype expression (Kelce & Wilson 1997). Another method of endocrine disruption is via hormone synthesis. Compounds can up- or down-regulate key enzymes involved in steroid synthesis. Some can stimulate the synthesis of estrogens by up-regulating aromatase and other key steroidogenic enzymes (Younglai et al. 2004; Whitehead & Rice 2006). EDCs can cause a range of developmental disorders from precocious puberty and an increased incidence of breast cancer in females to demasculinisation and increased incidences of reproductive defects such as hypospadias and cryptorchidism in males (this effect has been observed only in animal studies) (Gray et al. 2001; Bhatt 2000). This is because the ratio of androgens to estrogens is essential in determining whether male or female traits are expressed in early development. This is due to the fact that the active AR can inhibit ER action and *vice versa* (Akingbemi 2003; Peters et al. 2009). Therefore, any decrease in testosterone activity is equivalent to an increase in estrogenic activity because less ER is inhibited. Because of this androgens suppress the growth of healthy and cancerous breast cells which grow in response to estrogen stimulation. Removal of this suppression could lead to an increased chance of developing breast cancer because any cancerous mutation in a breast cell is more likely to divide under estrogen

stimulation leading to tumour formation (Kelce & Wilson 1997). On top of this some EDCs have a multigenerational effects (Dolinoy 2008) meaning these effects could take place during the early development of successive generations. Reproductive EDCs can cause a variety of detrimental effects via a variety of mechanisms, on top of this many of them are common environmental contaminants and some are still in use (for specific examples see section 1.2.2.). Identifying these EDCs and characterising their mechanisms of toxicity is the first step in ensuring that appropriate regulatory standards are in place. Based on what effects EDCs have on hormone synthesis, enzyme expression, and what these effects will have in the long-term will all contribute towards regulatory decisions. Informed regulatory decisions will result in the appropriate exposure limitations being implemented decreasing overall exposure to levels with no adverse effects.

4.1.3. The Effects of Dibutyl Phthalate and Other Phthalate Esters

DBP (along with other phthalates) is used as a plasticiser, stabiliser and pesticide in a variety of products, including cosmetics, inks, food packaging, adhesives and acaricides making it a ubiquitous environmental and food contaminant (Foster et al. 2000; Xiao-feng et al. 2009; Janjua et al. 2007; Carran & Shaw 2012). DBP is a known EDC and causes reproductive defects (e.g. hypospadias) in multiple animals across a broad evolutionary spectrum, including frogs (*Rana rugosa*) and rats (Mankidy et al. 2013; Pant et al. 2011; Ohtani et al. 2000; Wolf et al. 1999; Parks et al. 2000). Exposure of pregnant rats to phthalates (particularly DBP) resulted in their male offspring having reduced testosterone levels compared to controls (Howdeshell et al. 2008). How phthalates cause this decrease in testosterone has been the subject of discussion for many years. Some propose that direct inhibition of key steroidogenic enzymes is responsible (Yuan et al. 2012); whilst others

suggest that an epigenetic mechanism involving gene expression resulting in altered enzyme activity is responsible (Martinez-Arguelles et al. 2013).

Human exposure to phthalates is widespread due to the extensive use of these compounds. However, the implications of phthalate exposure on human health are difficult to determine because humans are exposed to multiple EDCs with common endpoints (e.g. male reproductive malformations). Recently Carran and Shaw (2012) showed a statistically significant increase in the incidence of hypospadias and cryptorchidism in the children of soldiers exposed to high levels of DBP (used as an acaricide to kill Trombiculid mites that carry bush typhus) (Carran & Shaw 2012; Elwood & Borman 2012). This suggests that DBP could have a multigenerational effect. However, most animal studies have only been exposed whilst pregnant resulting in the embryo receiving direct exposure to DBP rather than looking at long term multigenerational effects. These effects could be due to either mutagenic changes or epigenetic changes. DBP has been shown to be mildly mutagenic (Seed 1982; Kleinsasser et al. 2001). However, this is most likely general mutagenicity and would not necessarily result in multigenerational endocrine disruption. Another possibility is that DBP's effects are multigenerational via an epigenetic gene regulatory mechanism. This type of mechanism has been seen in other EDCs. BPA causes multigenerational epigenetic effects via alterations of DNA methylation patterns resulting in changed gene expression (Dolinoy 2008). It has been shown that BPA increases the expression of genes coding for DNA methyltransferase enzymes (DNMT1 and DNMT3A specifically) these enzymes methylate specific DNA residues. This paralleled an increase in ERα expression associated with methylation changes in the ERα gene (Kundakovic et al. 2013). Exposing a generation of rats to a combination of DBP, DEHP and BPA caused an increased incidence of testis disease three generations later (Manikkam et al. 2013). This could be due to BPA but the fact that there is an increased incidence of genital diseases in the soldier's children in Carran and

Shaw's study (Carran & Shaw 2012) suggests that DBP may have contributed to this effect; possibly via alterations of DNA methylation in genes responsible for the production of testosterone biosynthetic enzymes. This suggests that there is a significant multigenerational effect following DBP exposure. However, as only one subsequent generation was investigated in Carran and Shaw's study (Carran & Shaw 2012) whether this is a temporary change that will disappear after a few generations (i.e. it is an epigenetic effect) or whether this increased incidence persists (i.e. it is a mutagenic effect) is unknown.

4.2. Data from the Present Study

4.2.1. Characterisation of Cell Lines

Two Leydig cancer cell lines were the focus of this study. The first was the R2C cell line. R2C cells are spherical in shape and adhere to flasks when grown in culture. Once adhered, the spherical cells spread out becoming more oval shaped (Fig. 3.3A). When R2C cells are grown small conglomerates of cells visible to the naked eye, will begin to form by day 4, these cell conglomerates will continue to grow rapidly until they come into contact with each other and reach confluence and reach a maximum cell population of approximately 2 x 10^6 cells/mL (approx. day 8). By this point cells will cease growing and begin to die due to overpopulation (Fig. 3.2). When R2C cells are exposed to concentrated levels of DBP the morphology and growth of R2C cells is negatively impacted (see section 4.2.2.). The R2C cell line was chosen for two reasons. Firstly, it had been shown that they constitutively expresses all of their steroidogenic enzymes making them able to produce steroids without having to be chronically stimulated by LH or a cAMP analogue (Freeman 1987). This allowed the analysis of DBP exposure's effects on steroid biosynthesis to be much easier because chronic stimulation of the cells with LH or DB-cAMP did not have to be accounted for. However, the downside to this constitutive expression is that any changes in testosterone

synthesis due to interference with LH and cAMP stimulation cannot be observed in the R2C cell line. Secondly, a recent BSc Honours report suggested that R2C cells produced testosterone (Carran 2010). This made R2C cells a good model for studying the effects of DBP exposure on testosterone synthesis. However, in the present study it was shown, whilst the R2C cell line synthesises large quantities of progesterone, testosterone was undetectable (Fig. 4.12). It has recently been confirmed that testosterone is being made by the R2C cells, but it is not made at a level that can be detected using HPLC analysis (Balbuena et al. 2013). This might, in turn, be explained by a decrease in the expression of CYP17 as this enzyme is necessary for testosterone synthesis. However, complete loss of this enzyme's functionality is unlikely as R2C cells are capable of synthesising cortisol which requires CYP17's 17α-hydroxylyase activity to produce a key intermediate, 17α-hydroxyprogesterone (Fig. 4.1). Alternatively, 17β-HSD could be inactive or its expression could be reduced leaving the R2C cells with the ability to make detectable levels of cortisol but not detectable levels of testosterone. Because cortisol can be synthesised by R2C cells at observable levels this is more likely. This meant the exposure studies carried out using the R2C cell line could not look at testosterone biosynthesis, but rather only changes in progesterone and cortisol biosynthesis (see section 3.9). This prompted an investigation into a new cell line that could produce quantifiable amounts of testosterone, namely the LC-540 Leydig cell line.

Like the R2Cs the LC-540 Leydig cell line is adherent. However, the LC-540 line has a much longer growth lag period of about 10 days before rapid growth begins to occur (compared to 5 days for the R2Cs) (Fig. 3.2). LC-540 cells are spherical when suspended in media but once adhered they become very spread-out and do not form visible cell conglomerates. Instead, by day 11 LC-540 cells will form a monolayer which can only be identified under a 100x magnification and even then the individual cells are so spread out it is sometimes difficult to see their membranes (Figs. 3.2, 3.3B). By day 13 growth ceased and dead cells were seen

suspended in media (Fig 3.2.). By day 17 the monolayer began to peel off the surface of the flask (Fig. 3.2). It is interesting to see that even though both of these cell lines were isolated from Leydig cell tumours they differ greatly in both morphology and growth rate. They also differ in their steroid biochemistry. This was shown when LC-540 media extracts contained quantifiable levels of testosterone and progesterone (Fig. 3.13B). This is supported by the literature, LC-540 cells are known to synthesise and secrete testosterone and 17β-estradiol (Steinberger et al. 1970) (Fig. 4.1). This means that LC-540 cells express all of the enzymes necessary to synthesise testosterone and have not lost their ability to express these enzymes in their active forms. The expression levels of genes coding for essential steroidogenic enzymes in LC-540 cells seem to be regulated by internal cAMP concentration. This was hinted at in the present study when exposure of LC-540 cells to 1mM of the cAMP analogue DB-cAMP seemed to stimulated steroidogenesis (see section 3.10.). Unbutylated cAMP however, did not stimulate steroid synthesis (see section 3.10.). This is most likely due to cAMP being unable to pass through the cell membrane due to cAMPs highly hydrophilic structure. This suggests that LC-540 cells may have retained the proteins responsible for cAMP induced expression of the genes coding for steroidogenic enzymes (Gyles et al. 2001). However, it is unknown whether LC-540 cells respond to pituitary peptide hormones (such as LH) like isolated Leydig cells do (Ge & Hardy 1998). The regulatory action of cAMP on the LC-540 cell line has not been investigated previously. However, even without stimulation, LC-540 cells synthesise quantifiable amounts of testosterone. The combination of the LC-540 cell's ability to synthesise testosterone along with the potential retention of the internal cAMP stimulatory response makes this cell line a much better model for the assessment of a compounds effects on steroidogenesis than the R2C cell line with their undetectable testosterone biosynthesis and lack of a cAMP steroidogenesis regulatory mechanism. Therefore LC-540 cells would be ideal as a model to investigate the effects of DBP on

testosterone biosynthesis. However, due to time constraints and difficulty setting up LC-540 cell cultures only R2C cells could be exposed to DBP and have their steroid secretions analysed.

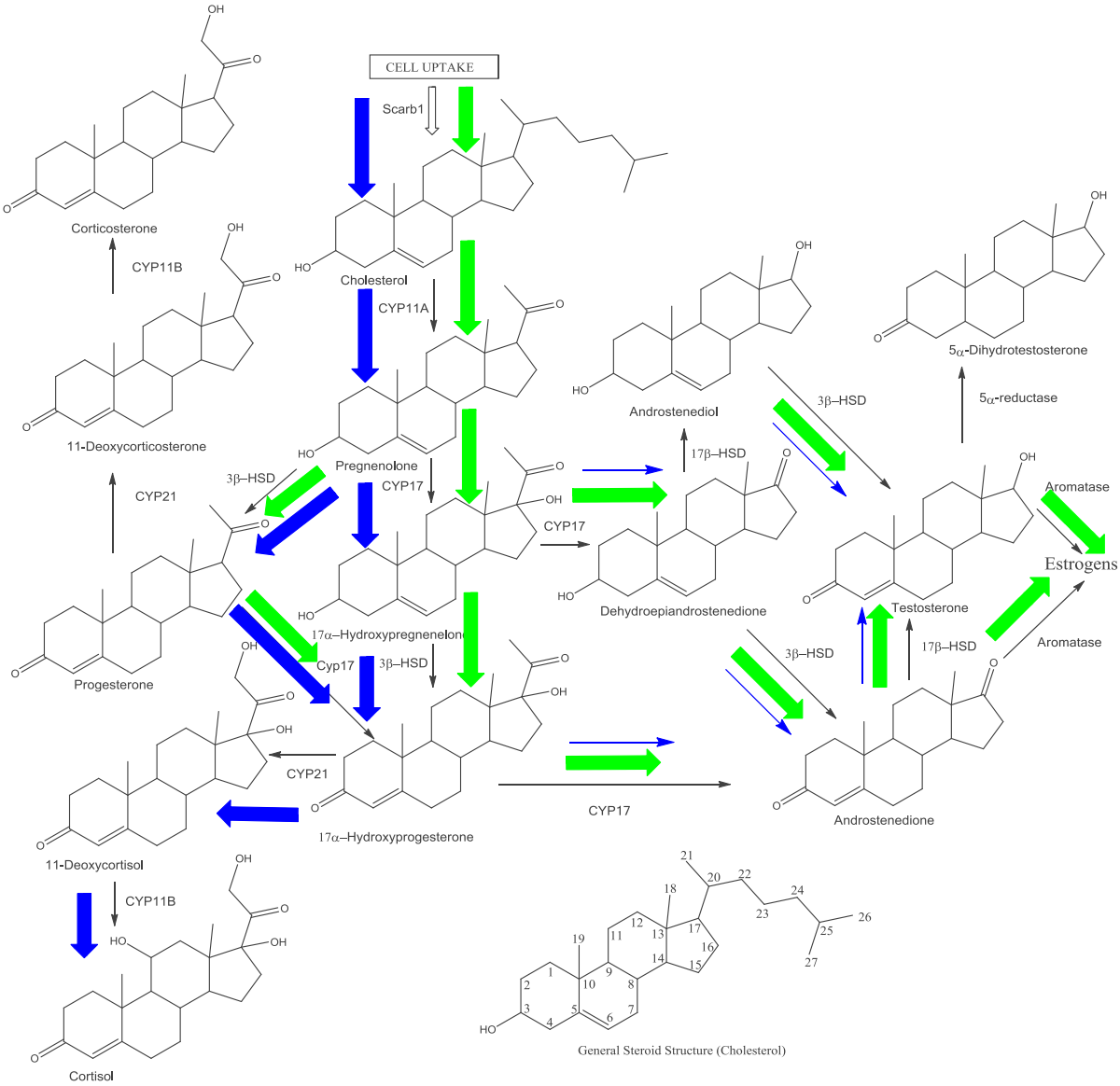

Figure 4.1: Steroidogenesis pathway showing which constituents of the pathway each cell line is capable of synthesising. The R2C cells are capable of synthesising Cortisol when stimulated and LC-540 cells are known to synthesise testosterone and 17β-estradiol (Steinberger et al. 1970). ▶ = LC-540 steroidogenesis activity, ▶ = R2C steroidogenesis activity. Small blue arrows indicate documented testosterone synthesis that was undetected in the current study (Balbuena et al. 2013).

4.2.2. Determination of Relevant DBP Exposure Concentrations

Exposure of R2C cells to 0.297 and 2.44 mg/mL DBP resulted in almost 100% cell death as cell concentrations in these samples were significantly lower than control levels, whilst a DBP exposure level of 8.6×10^{-5} mg/mL caused no observable cell death (Table 3). The cell death seen at very high DBP exposure levels was likely caused by the formation of insoluble DBP globules in the culture medium due to DBP oversaturation; this was seen in high DBP exposure samples (Fig. 3.14B, C). Because DBP has an extremely high octanol/water partition coefficient (LgPow) (between 4.31 and 4.79) (WHO 1997) it is possible that cells that came into contact with the globules rapidly absorbed large quantities of DBP into their membranes. This in turn could lead to membrane property changes akin to those seen when cholesterol levels in membranes are high. High membrane cholesterol levels result in changes in phospholipid R-group packing and this alters membrane fluidity. DBP might act in a similar way because it too is likely to dissolve in the phospholipid R-groups disrupting membrane packing in a manner similar to cholesterol (Gleason et al. 1991). This will alter the membranes fluidity disrupting normal membrane function which in turn will lead to the loss of surface protein functionality (Fig. 4.2). This could explain the massive loss in cell number at high exposure levels.

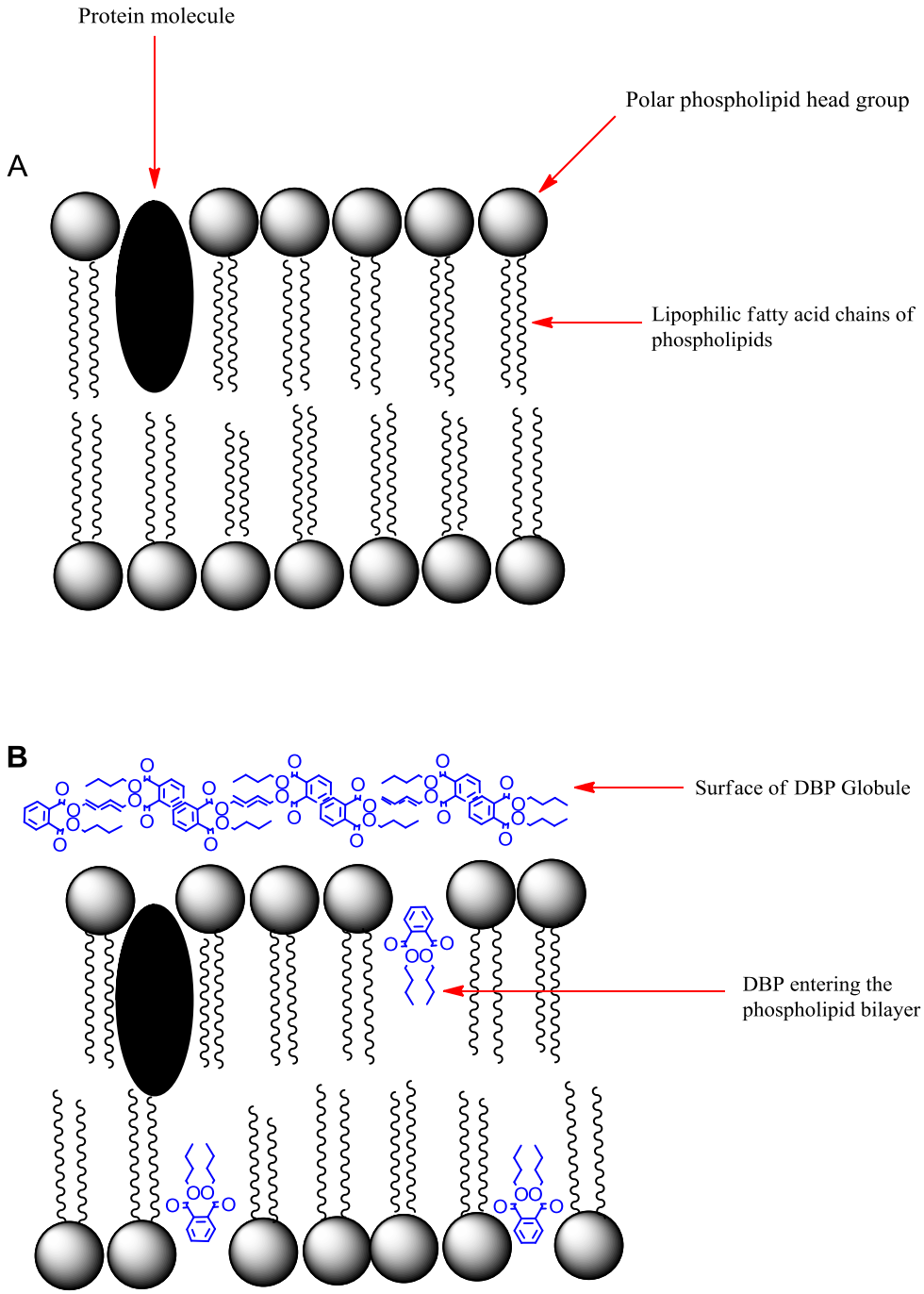

Figure 4.2: Potential mechanism of toxicity of DBP at levels insoluble in culture medium. Large quantities of DBP might enter the cell membrane when a cell comes in contact with an insoluble globule. This could lead to alterations in membrane fluidity due to changes in phospholipid packing disrupting the membrane and its protein's function. ***A:*** *Cell membrane structure.* ***B:*** *A disrupted cell membrane exposed to a concentrated globule of DBP.*

These effects are less likely to been seen *in vivo*. This is because DBP is rapidly metabolised to MBP by multiple esterases. These esterases are most active in the liver (Hanioka et al.

2012). However, many non-specific esterases are produced throughout the body (including Leydig cells). These non-specific esterases can also catalyse DBP hydrolysis (Molenaar et al. 1986) both *in vivo* and *in vitro*. The *in vivo* metabolism of DBP is, in fact, so rapid that no DBP was detected in the plasma and urine of rats dosed with 100 mg/kg body weight [^{14}C]-DBP. MBP and MBP glucuronide were instead the two major components found in both urine and serum within two hours of administration (Fennell et al. 2004). However, there will be no first pass effect through the gut when DBP is applied topically as in Carran and Shaw's DBP exposed soldiers (Carran & Shaw 2012) as DBP will be directly absorbed through the skin into the blood. But, due to the high concentration of non-specific esterases in the skin (Montagna 1955) DBP is likely going to be mostly hydrolysed to MBP before it reaches the blood. Because MBP has lost one of its butyl groups and has gained a carboxylic acid hydroxyl group (which can be deprotonated and delocalise its negative charge with the other carboxylic acid oxygen) this will make it much more water soluble than DBP meaning less insoluble globules will form in the blood. It has been suggested that MBP is responsible for DBP's endocrine-disrupting effects (Clewell et al. 2010). The majority of a DBP dose was excreted within 24 hours of administration (Fennell et al. 2004) this removal of DBP via excretion is obviously impossible during an *in vitro* exposure. Considering most *in vivo* studies of phthalates are either via oral (Howdeshell et al. 2008; Howdeshell et al. 2007; Parks et al. 2000) or skin exposure (Carran & Shaw 2012; Carran & Shaw 2010) the skin and liver will metabolise a significant percentage of the DBP prior to it reaching the testis and Leydig cells. This means physical toxicity such as the effects seen at 2.44 and 0.297 mg/mL doses are unlikely to occur *in vivo*. Therefore these concentrations are inappropriate for further analysis of DBP's effects on steroid synthesis.

There were no significant changes in cortisol production at DBP exposure levels 8.6×10^{-5} and 0.294 mg/mL (Fig. 3.15). Cortisol production was below the LoD at DBP exposure level

2.44 mg/mL and sample noise was much greater than the control at DBP exposure level 0.294 mg/mL (Fig. 3.15). Progesterone decreased significantly ($P < 0.05$ when compared to control) at both 0.294 and 2.44 mg/mL DBP doses (Fig. 3.16). However, these apparent decreases in steroid production are likely due to inaccuracies in cell count. Particularly the samples exposed to 2.44 mg/mL DBP have cell concentrations of approximately 1×10^4 cells/mL this is well out of the accurate range (1×10^4 cells/mL corresponds to one cell on the haemocytometer). Due to this inaccuracy, it is much more likely that the actual cell concentration is less than that counted. Because of this, when the steroid concentrations were normalised, the overestimated cell concentration resulted in an apparent overall decrease in steroids concentration produced on a per cell basis. Alternatively, cells could be down regulating the steroid pathway to conserve metabolic energy for vital pathways in response to a general toxic insult. Regardless of these inaccuracies, these concentrations are inappropriate for looking at the biochemical effects of DBP on Leydig cells. This was either due to the absence of an observable effect (as seen in the samples exposed to 8.6×10^{-5} mg/mL DBP) or due to the toxic insult losing its specificity and causing a physical toxic effect (seen in the samples exposed to 0.297 and 2.44 mg/mL DBP). To remedy this, different concentrations were studied that were below DBP's water saturation point (to avoid the physical toxicological effects of DBP globules on cells) and above the unresponsive low dose.

4.2.3. Effects of DBP Exposure on R2C Cell Steroid Biosynthesis

Exposure of R2C cells to DBP resulted in the induction of cortisol production at all exposure levels (0.1-10 μg/mL) compared to controls ($P < 0.05$) (Fig. 3.17). Whilst cortisol production does not significantly change between exposed samples the overall trend suggests that cortisol production increases with DBP dose (Fig. 3.17). This effect has not been reported previously and is particularly significant because Leydig cells are not known to express the

necessary enzymes for glucocorticoid synthesis (CYP21 and CYP11B1) (Payne & Hales 2004). The effects of activating cortisol biosynthesis are likely to be very significant, particularly during early growth and development. This is because cortisol is produced from the same precursors as testosterone (Fig. 1.6). Therefore, initiating cortisol production could starve testosterone biosynthesis of precursors (Fig. 4.4). Glucocorticoids such as cortisol are known to have a negative impact on Leydig cell growth and can potentially induce Leydig cell apoptosis as well as down regulating steroid biosynthetic enzymes in Leydig cells further reducing testosterone production (Gao et al. 2002; Consten et al. 2002; Drake et al. 2009). This could result in an overall decrease in serum testosterone which during early development could cause genital malformations such as cryptorchidism and hypospadias because testosterone is essential for Wolffian duct development and testis descent.

Progesterone production, on the other hand, does not change significantly ($P < 0.05$ compared to control) in response to DBP exposure (Fig. 3.16). This suggests that DBP does not affect the early stages of steroidogenesis. However, this could be due to the fact that R2C cells are insensitive to both pituitary peptide hormones (e.g. LH) and internal cAMP concentration (both of which effect the early stages of steroidogenesis); indeed it has been shown that progesterone concentration decreases in cultured mouse MA-10 Leydig tumour cells (these cells have their LH stimulation pathway intact) in response to MBP exposure (Clewell et al. 2010). Clewell et al (2010) also showed that another phthalate monoester (MEHP) causes down regulation of early steroidogenic enzymes (including StAR, CYP11A, CYP17), this suggests that DBP (once it has been metabolised to MBP) could also affect the early enzymes in the steroidogenesis pathway via interference with LH binding or internal cAMP stimulation thus preventing up regulation of these enzymes which could result in reduced production of testosterone by Leydig cells (Fig. 4.3). This could be a potential mechanism for DBP toxicity.

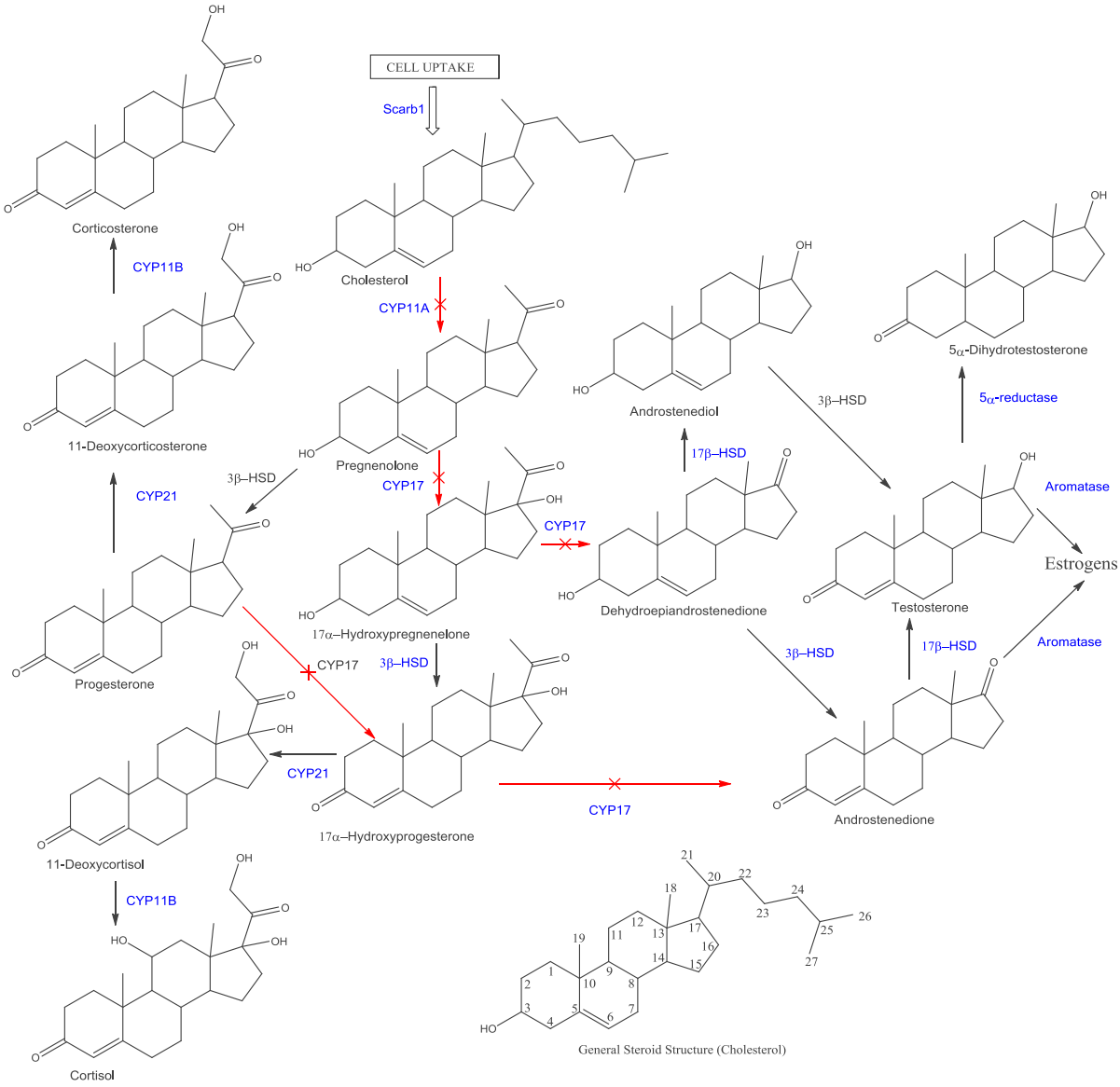

Figure 4.3: Steroidogenesis pathway showing the reduction in StAR, CYP11A, and CYP17 expression demonstrated by Clewell et al (2010) on the steroidogenic pathway. Enzyme catalysis of the steps highlighted in red will be significantly reduced compared to Leydig cells under LH stimulation. This will starve the later stages of testosterone synthesis of enzymes. This will result in reduced testosterone secretions by Leydig cells. → = active enzyme pathways. ~~X~~> = Pathways with reduced enzyme activity due to the suppression of LH stimulation.

Another possible mechanism of DBP toxicity is its direct inhibition of steroidogenic enzymes. Kinetic studies of both human and rat 3β-HSD and 17β-HSD have shown that several phthalates (including DBP) directly inhibited these enzymes activity in a concentration dependant manner (Yuan et al. 2012). However, Yuan et al (2012) also

demonstrated that the monoester metabolites of these phthalates (e.g. MBP) had little or no effect on enzyme catalysis (e.g. MBP had no effect on human 17β-HSD even at mM concentrations). Therefore, it is unlikely that direct steroidogenic enzyme inhibition is a possible mechanism since DBP is extensively and rapidly metabolised by esterases to MBP *in vivo* (Hanioka et al. 2012; Fennell et al. 2004) (Fig. 1.10).

The data discussed above suggests a new mechanism of toxicity for DBP, since cortisol synthesis uses the same metabolic precursors as testosterone synthesis an increase in cortisol synthesis could result in a decrease in testosterone synthesis (Fig. 4.4) (this mechanism of toxicity would be augmented if metabolic precursor (e.g. progesterone) biosynthesis is slowed by DBP interfering with LH stimulation as observed in Clewell et al's (2010) study). This change in metabolic flux might result in an overall decrease in circulating testosterone levels. In addition, glucocorticoids have been shown to cause negative effects on Leydig cell development and are known to induce apoptosis in Leydig cells (Gao et al. 2002; Consten et al. 2002). Glucocorticoids have also been shown to decrease androgen production and even amplify the effects of phthalates on male reproductive development (Drake et al. 2009; Consten et al. 2002) and thus further reduce testosterone synthesis. This could result in a feedback mechanism where DBP induces cortisol synthesis and that, in turn, amplifies DBPs effect further reducing testosterone synthesis. This increase in cortisol biosynthesis must be caused by up-regulation of the enzymes CYP11B and CYP21 by DBP as these enzymes are essential for glucocorticoid synthesis and are not normally expressed in Leydig cells (Fig. 4.4). This is supported by recent data showing that R2C cell's testosterone production is decreased by DBP in a dose dependant manner (Balbuena et al. 2013). Because R2C cells are not stimulated by LH (as well as other pituitary peptide hormones) reductions in LH stimulation will not cause a testosterone decrease. This means the only possible mechanisms of toxicity must influence enzyme activity in other ways, potentially via cortisol biosynthesis.

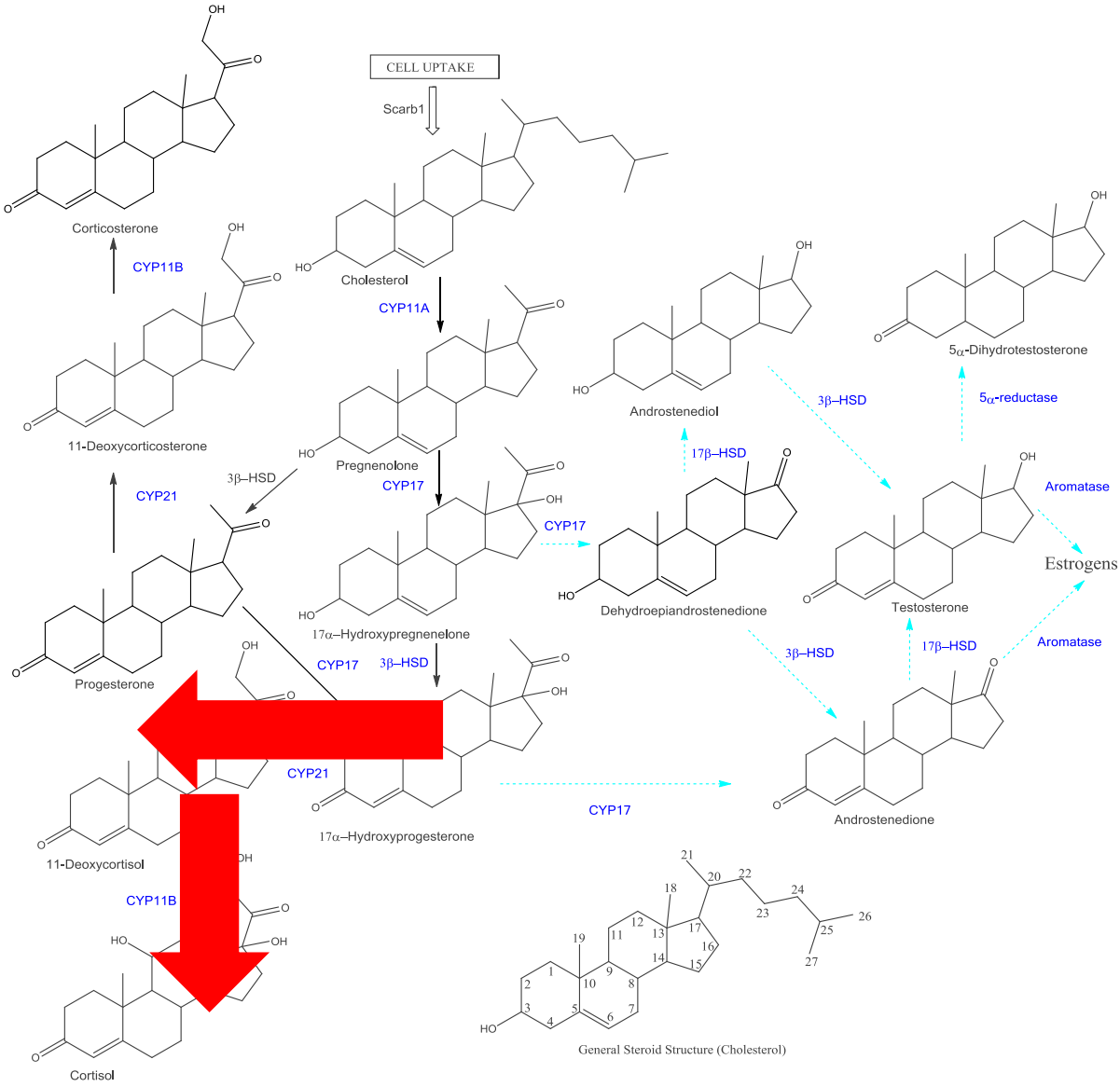

Figure 4.4: Steroidogenesis pathway showing the effect of elevated cortisol biosynthesis on the steroidogenic flux in Leydig cells. Elevated cortisol biosynthesis will result in the starvation of precursors for androgen biosynthetic processes. ▶ =Stimulated cortisol biosynthetic metabolic steps ⇢ = Androgen biosynthetic processes that will compete for precursors.

A combination of these mechanisms of toxicity could explain how exposure to DBP (and other phthalates) would result in decreased serum testosterone levels. As testosterone is vital for development of the Wolffian duct, external genitalia, and descent of the testis, this would explain how direct *in utero* phthalate exposure might result in developmental defects such as cryptorchidism and hypospadias (Parks et al. 2000; Howdeshell et al. 2007; Howdeshell et al.

2008; Foster et al. 2000). However, it has recently been shown that the children of New Zealand soldiers exposed to high levels of DBP during their service in the Malayan Emergency have a higher incidence of cryptorchidism and hypospadias than the general population (Carran & Shaw 2010; Carran & Shaw 2012), this suggests that DBP's effects are multigenerational. This in turn, suggests that DBP mediated toxicity is via an epigenetic gene regulatory mechanism because direct exposure of the developing embryo to DBP in this case is impossible. This means that maternal DBP exposure could have direct effects on the developing embryo's expression of genes coding for steroidogenic enzymes (if exposure occurs during pregnancy as in most rat based studies (Parks et al. 2000; Howdeshell et al. 2007; Howdeshell et al. 2008; Foster et al. 2000)) or the epigenetically-mediated changes could be inherited via the ovum. However, paternal DBP exposure can only be passed to the offspring by epigenetic changes in steroidogenic enzyme expression inherited via the sperm. Paternal epigenetic inheritance is known to influence an offspring's phenotype (Curley et al. 2011). For example, the EDC BPA has been shown to have multigenerational epigenetic effects via alterations in DNA methylation patterns in multiple mouse models (Dolinoy 2008; Kundakovic et al. 2013). These alterations have been shown to cause estrogenic effects (Kundakovic et al. 2013). DBP could be inducing an epigenetic effect analogous to this. In another study it was shown that a similar effect occurred in rats exposed to a mixture of BPA, DBP, and DEHP (Manikkam et al. 2013). This suggests that DBP might induce an epigenetic change in the sperm that could be transferred to the developing embryo resulting in reproductive defects such as cryptorchidism and hypospadias.

An important question to ask is, are these effects likely to occur in the general population? This is difficult to determine because MBP levels in human serum have not been quantitatively measured. The lowest DBP exposure level that initiated cortisol synthesis was approximately 0.36 μM (0.1 μg/mL) and the lowest MBP doses that induced a decrease in

steroidogenesis as shown by Balbuena et al (2013) (showed a testosterone decrease in R2C cells) and Clewell et al (2010) (showed a decrease of LH stimulation in MA-10 cells) were 1 μM and 3 μM respectively. The European Food Safety Authority found that adults in Denmark were exposed to dietary DBP doses of 4.1 μg/kg body weight/day (EFSA 2005). Assuming that the average male weighs about 70kg, has 3.3L of serum and 100% absorption the final serum concentration is 0.3μM. However, before entering the serum DBP is rapidly and completely metabolised to MBP which has been shown in humans to have a conversion rate of 69% (Anderson et al. 2001) and is completely absorbed into the circulatory system due to its low volume of distribution (Kremer et al. 2005). This would mean that there will be a total serum concentration of approximately 0.2 μM. However, all of the MBP will not enter the serum simultaneously and using this calculation for the average rat (weight of 400 g and 64 mL/kg serum) being dosed with 100 mg/kg body weight DBP this calculation suggests that the total serum levels of MBP should be 1.5 mM. But, pregnant dams given a radiolabelled dose of DBP had an average peak MBP serum level of 329 μM as well as their fetus having an average peak dose of 182 μM an order of magnitude lower than the calculated concentration. On top of this MBP will also be glucuronidated and human urine studies have shown that of a total urine MBP concentration of 29.7 mg/L only 1.4 mg/L was free MBP the rest was glucuronidated (Silva et al. 2003). This means that the DBP and MBP concentrations in the *in vitro* studies above are likely much higher than what the general population is exposed to. However, this does not mean that the phthalate doses the general population are exposed to will not have an effect. Swan et al. (2005) found that AGD decreased in correlation with increased prenatal phthalate exposure and that ¼ of the American female population were above the median phthalate levels associated with decreased AGD and increased cryptorchidism. This suggests that environmental levels of phthalates do have an impact on reproductive development. It has also been suggested that phthalates are acting in

tandem with other anti-androgenic and estrogenic EDCs that act through a variety of mechanisms but all influence the androgen-estrogen ratio causing an overall decrease in serum testosterone and an increased incidence of male developmental disorders such as cryptorchidism (Toppari et al. 2006). There are also groups who are exposed to elevated doses of phthalates through their work. A cohort of construction workers regularly exposed to high levels of DBP and DEHP had much higher urine concentrations of MBP and MEHP and significantly lower free testosterone concentration compared to unexposed construction workers (Pan et al. 2006). Another example would be the Malayan Emergency veterans studied by Carran & Shaw (2012) who were exposed to a theoretical dose above the no observable adverse effect level (NOAEL). These groups are more likely to have serum levels that equate to the DBP and MBP concentrations used in *in vitro* studies and are therefore more likely to have similar alterations in their Leydig cell's steroid production (i.e. decreased testosterone production and increased cortisol production).

In conclusion DBP causes an increase in cortisol production in R2C Leydig cells. This could alter the steroidogenic flux toward cortisol and away from testosterone. The cortisol produced could then adversely affect Leydig cell development further decreasing testosterone production. If this mechanism occurs *in vivo* along with the previously documented ability of DBP to inhibit LH stimulation this could explain how DBP exposure leads to male developmental defects such as cryptorchidism and hypospadias.

4.3 Future Work

To further support this hypothesis, further studies could be performed with both R2C and LC-540 cell lines using the much more hormone specific radioimmunoassay technique. This would produce much more accurate steroid media level data and will eliminate background noise caused my other compounds found in the media extract. DB-cAMP's effect on LC-540

steroidogenesis could be looked at in more depth to determine whether the LC-540 cell line has the early regulatory mechanisms for steroidogenesis seen in wild type Leydig cells. The LC-540 cell line could be used in similar experiments to the R2C cell line. Because it can produce quantifiable levels of testosterone it could be used to compare cortisol and testosterone production during DBP exposure. CYP21, CYP11B, and 17β-HSD expression could also be measured to show if the cortisol synthesising enzymes (CYP11B and CYP21) are up regulated and if 17β-HSD (an enzyme used exclusively for androgen and estrogen synthesis) is down regulated.

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